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COMPARATIVE ACTIVITIES OF URINARY GONADOTROPIN PREPARATIONS FROM POSTMENOPAUSAL WOMEN*

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ABSTRACT

The gonadotropic activities of 4 preparations of human pituitary gonadotropin (HPG) from postmenopausal urine (Pergonal, HMG-20A, HMG-24, and HMG-J5), obtained by different techniques of extraction and purification, were compared with an HPG preparation designated as PM, obtained from the same source by the kaolin-acetone extraction procedure. Four bioassay methods were used. The mean relative potencies of Pergonal and HMG-J5 in terms of PM did not differ significantly in any of the biologic tests used. The mean relative potency of HMG-20A in terms of PM varied according to the bioassay method employed. All assays were valid with regard to the statistical tests applied. In particular, there was no evidence of significant slope differences in any of the assays. It was concluded that, although qualitative differences may have existed among the preparations tested, each of the preparations could have been used to express the gonadotropic potency of the others by means of the assays employed in this study.

THE establishment of an international reference preparation for human urinary gonadotropins (HGP) has been extensively discussed in the literature (1–7). Several preparations of HPG obtained by various workers from the urine of postmenopausal women and from normal men by differ-

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ent techniques of extraction and purification have been studied. Their relative gonadotropic potencies in terms of the preparations designated as HMG-20A and HMG-24 [International Reference Preparation—IRP—for Human Menopausal Gonadotrophin (8)] made by Organon Laboratories Ltd., Lanarkshire, Scotland, were estimated by various end-points. Differing potency ratios were observed and it was concluded that HMG-20A and HMG-24 varied qualitatively from the variety of extracts tested. It was inferred that the reference material should be prepared by substantially the same technique of extraction and purification as the gonadotropic material to be assayed.

The present study was undertaken to compare, in several biologic systems, the various preparations of HPG obtained by different extraction procedures from postmenopausal urine with that obtained by the kaolin-acetone procedure (9) from the same source.

MATERIAL AND METHODS

Urinary extracts

Five extracts obtained from postmenopausal urine were used: **1**) PM, local laboratory standard prepared by the kaolin-acetone procedure (9). **2**) HMG-20A, prepared by the kaolin-acetone method including tricalcium phosphate purification (1). **3**) HMG-24, prepared as HMG-20A followed by fractionation with ammonium acetate-ethanol mixtures (10). **4**) HMG-J5, extracted and purified by a chromatographic method, employing zeolite as the adsorbent material (11). **5**) Pergonal, extracted and purified by a chromatographic method, employing Permutit as the adsorbent material (12).

Methods of assay

The bioassay methods used were the following: (*a*) the uterine weight test in intact immature female mice; (*b*) the ovarian weight test in female rats treated with 20 I. U. of HCG; (*c*) the testicular and ventral prostate weight test in immature hypophysectomized male rats.

These methods have been well described and used by many workers in the field and require no further elaboration of technical details (13–15). The number of replicate assays conducted on any given end-point was dependent on the amount of available material. In the ovarian weight test (HCG-treated female rats), HMG-24 was used owing to the scarcity of HMG-20A.

Statistical evaluation of the data was conducted according to the methods of Fisher (16), Bülbring (17), Bliss (18); Emmens (19) and Gaddum (20). In all assays, validity tests were satisfactory. In particular, there was no significant deviation from parallelism in any assay ($P > 0.5$).

Sensitivity of end-points

The relative sensitivities of the four end-points used in this study are shown in Table 1.

The amount of each preparation required to produce a response differing from the mean value observed at zero dose by three times the standard deviation of the controls was considered the minimal effective dose.

TABLE 1. SENSITIVITY OF END-POINTS ASSAYING DIFFERENT PREPARATIONS OF HPG

End-point	Amt. of preparation used (mg.)				Sensitivity			
	PM	Pergonal	HMG-J5	HMG-20A	PM	Pergonal	HMG-J5	HMG-20A
Testicular weight (hypophysectomized rats)	2.6	0.08	0.06	1.7	1	1	1	1
Ventral prostate weight (hypophysectomized rats)	2.5	0.11	0.12	1.9	1	0.73	0.5	0.9
Uterine weight (immature mice)	0.6	0.02	0.02	0.4	4.3	4.0	3.0	4.3
Ovarian weight (HCG-treated rats)	3.6	0.18	0.10	16	0.7	0.4	0.6	0.11

Assigning a value of 1 for the sensitivity of the testicular weight method, the sensitivities of the other three were obtained by dividing the minimal effective dose on each end-point into the minimal effective dose on the testicular weight assay.

Potency of preparations

The relative potencies of Pergonal, HMG-J5 and HMG-20A in terms of PM are shown in Table 2.

RESULTS

Uterine weight assay in intact immature female mice

Pergonal. Four assays were performed. The mean relative potency (R.P.) was 31.9. There was complete overlap in confidence limits, that is, the upper confidence limit of all 4 assays was no less than the lower confidence limit of any of the assays. The index of precision ranged from 0.08 to 0.14. The degree of internal uniformity was satisfactory with a variance ratio of $P(F) > 0.5$.

HMG-J5. Three assays were performed. The mean R.P. was 36.9. One of the upper confidence limits was less than the lower confidence limit of one of the assays. The index of precision ranged from 0.06 to 0.17. The degree of internal uniformity was reasonably satisfactory with a variance ratio of $P(F) > 0.01 < 0.05$.

HMG-20A. Two assays were performed. The mean R.P. was 1.32. There was complete overlap in confidence limits. The index of precision varied from 0.12 to 0.19. The degree of internal uniformity was satisfactory with a variance ratio of $P(F) > 0.05 < 0.5$.

Ovarian weight assay in HCG-treated immature female rats

Pergonal. Three assays were performed. The mean R.P. was 20.8. This value was lower than that obtained with the mouse uterus test. There was

TABLE 2. ASSAY OF PERGONAL, HMG-J5 AND HMG-20A IN TERMS OF PM

End-point	Material tested	N*	Design†	λ‡	R.P.§	Confidence limits¶	Mean**
Uterine weight (immature mice)	Pergonal	34	2×2	0.08	35.7	31.4–40.6	
		41	3×2	0.09	35.8	31.4–40.9	31.9
		35	3×2	0.14	28.3	22.7–35.3	
		34	3×3	0.09	28.5	24.7–32.9	
	HMG-J5	35	2×2	0.17	46.7	35.5–61.2	
		31	2×2	0.06	30.5	27.6–33.7	36.9
		35	3×3	0.13	35.4	28.9–43.3	
	HMG-20A	38	2×3	0.19	1.22	0.92–1.62	1.32
		34	3×3	0.12	1.43	1.18–1.73	
Ovarian weight (HCG-treated rats)	Pergonal	19	2×2	0.13	18.9	14.3–24.9	
		19	2×2	0.10	23.8	19.3–29.4	20.8
		18	2×2	0.13	19.9	15.2–26.0	
	HMG-J5	17	2×2	0.13	36.7	27.4–49.1	35.9
		19	2×2	0.10	35.2	28.5–43.5	
	HMG-20A*	16	2×2	0.14	0.22	0.16–0.30	0.22
Testicular weight (hypophysectomized rats)	Pergonal	16	2×2	0.18	23.2	12.2–44.1	23.2
	HMG-J5	16	2×2	0.28	36.9	19.4–70.3	36.9
	HMG-20A	16	2×2	0.34	0.70	0.32–1.53	0.70
Ventral prostate weight (hypophysectomized rats)	Pergonal	16	2×2	0.26	31.9	17.6–58.0	31.9
	HMG-J5	16	2×2	0.20	34.0	21.5–53.9	34.0
	HMG-20A	16	2×2	0.17	2.05	1.39–3.03	2.05

* Number of animals.
† Number of dose levels of unknown and standard.
‡ Index of precision.
§ Relative potency or units per mg., 1 unit being the activity of 1 mg. of PM.
¶ Calculated from assays with IRP (HMG-24) as a standard, assuming the potency of the latter as 0.8 (uterine weight in mice) (21); P = 0.95.
** Geometric means of the replicate results.

complete overlap in confidence limits. The index of precision ranged from 0.10 to 0.13. The degree of internal uniformity judged by the variance ratio P (F) $>0.05<0.5$ was satisfactory.

HMG-J5. Two assays were performed. The mean R.P. was 35.9. This value agreed well with that obtained in the mouse uterine test. There was complete overlap in confidence limits. The index of precision varied from 0.10 to 0.13. The variance ratio P(F) >0.5 was satisfactory.

HMG-20A. Due to the lack of HMG-20A for performance of this assay, HMG-24 was used instead. The biologic similarity of these two preparations was discussed at the "Workshop Conference on Human Pituitary Gonadotropins," Gatlinburg, Tennessee (21); consequently, the substitution was considered valid. The R.P. value of HMG-20A was calculated

assuming that 1 mg. of HMG-24 is equivalent to 0.8 mg. of HMG-20A. The calculated R.P. value of 0.22 was much lower than that obtained with the mouse uterine weight assay. The index of precision was 0.14.

Testicular weight assay in hypophysectomized immature male rats

Pergonal. In one assay the estimate of R.P. was 23.2. This agreed with results obtained by the ovarian weight test. The index of precision was 0.18.

HMG-J5. In one assay the estimate of R.P. was 36.9. This value agreed well with those obtained by the mouse uterine and ovarian weight tests. The index of precision was 0.28.

HMG-20A. In one assay the estimated R.P. was 0.70. This value did not agree with those obtained by the mouse uterus and ovarian weight test. The index of precision was 0.34.

Ventral prostate weight assay in hypophysectomized immature male rats

Pergonal. In one assay the estimated R.P. was 31.9. This value agreed with that obtained by the mouse uterine weight test. The index of precision was 0.26.

HMG-J5. In one assay the estimated R.P. was 34.0. This value agreed with those obtained by the mouse uterine weight, ovarian weight and testicular weight tests. The index of precision was 0.20.

HMG-20A. In one assay the estimated R.P. was 2.05. This value was much higher than those obtained by the mouse uterus, ovarian weight and testicular weight assays. The index of precision was 0.17.

Combination assays

Table 3 shows the index of discrimination for all combinations of assays. The statistical significance of the index of discrimination was tested by

TABLE 3. MEAN INDEX OF DISCRIMINATION FOR ALL COMBINATIONS OF ASSAYS

Combination of assays	No.	Index of discrimination (preparation)		
		Pergonal	HMG-J5	HMG-20A
Uterus (mouse) Prostate (rat)	1	1.00	1.09	0.64
Uterus (mouse) Testicles (rat)	2	1.38	1.00	1.89
Uterus (mouse) Ovary (rat)*	3	1.53	1.03	6.00
Testicles (rat) Prostate (rat)	4	0.73	1.09	0.34
Ovary (rat)* Testicles (rat)	5	0.90	0.97	0.31
Ovary (rat)* Prostate (rat)	6	0.65	1.06	0.11

* HCG-treated rats.

means of ordinary t tests dividing the difference of two log R.P.'s, each based on one or several estimates by the square root of the sum of the squared variances of the respective log R.P.'s. For Pergonal, the average index of discrimination for all combinations of assays did not differ significantly from unity, with the exception of index No. 3 (uterus/ovary) $P < 0.01$. HMG-J5 showed an average index of discrimination for all combinations of assays which did not differ significantly from unity. In the case of HMG-20A, indices No. 3, 4, 5 and 6 differed significantly from unity ($P < 0.05$).

COMMENTS

The present study was undertaken in an effort to investigate if several preparations of postmenopausal HPG obtained by various methods of extraction and purification could be assayed in terms of each other in each of 4 biologic assay systems. Of the end-points used, the ovarian weight in HCG-treated rats and the ventral prostate weight in hypophysectomized immature male rats are generally considered to measure specifically follicle-stimulating hormone (FSH) and luteinizing hormone (LH) respectively.

The estimation of the R.P. of HMG-J5 in terms of PM revealed no significant differences in any of the assays used. The mean R.P. for the 7 assays was 36.2 and the indices of discrimination for the various combinations of tests diverged little from unity.

Pergonal exhibited a somewhat lower R.P. in terms of PM with the ovarian weight and testicular weight assays as compared to the uterine weight and ventral prostate assays. The mean R.P. for the 9 assays performed was 26.6 and the mean indices of discrimination varied from 0.65 to 1.53.

Thus, both HMG-J5 and Pergonal were demonstrated to be: 1) more potent than PM, with HMG-J5 tending to be more potent than Pergonal; and 2) not significantly different biologically as judged by these tests. Albert *et al.* (22) also found Pergonal, HMG-J5 and a kaolin-acetone extract from postmenopausal urine, not to differ biologically when analyzing the parameters of dose-response curves with the rat ovarian-weight technic.

HMG-20A was more active than PM with the ventral prostate weight assay and least active with the ovarian weight test. Between these extreme values, the other two methods (mouse uterine weight and testicular weight) yielded potency ratios in an intermediate range. The mean indices of discrimination ranged from 0.11 to 6.0.

The R.P. of Pergonal in terms of HMG-20A calculated from Table 2, varied from 16 (ventral prostate weight test) to 94 (ovarian weight test); HMG-J5 showed a span in R.P. of 17 and 163 in these two tests, when analyzed in terms of HMG-20A.

The present observations confirm the evidence of dissimilarity noted with some of these preparations by other workers in the field (2–4, 6, 7, 23). The number of factors which are likely to produce evidence of dissimilarity when two preparations are compared in different types of bioassays have been reviewed by Albert and Rosemberg (24) and Borth, Linder and Lunenfeld (6), so it is not considered necessary to re-enumerate these factors here.

However, regardless of qualitative differences among the preparations studied, in the absence of significant slope differences in any of the tests within the error of these bioassays, each of these preparations could have been used as the reference standard to express the gonadotropic potency of the others in any of the end-points used. This indicates that clinical assays may be conducted in conjunction with a standard obtained by a different extraction procedure than that of the material under investigation. If, in fact, qualitative differences exist among urinary HPG preparations obtained by different extraction methods, it would be desirable to relate the results obtained in the estimation of potency to the end-point used for such purpose.

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THE USE OF CONTINUOUS INFUSIONS OF CALCIUM^{45} AND STRONTIUM^{85} TO STUDY SKELETAL FUNCTION

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ABSTRACT

Intravenous infusions of calcium^{45} of high specific activity were given for 1.7 to 3.3 hours at constant rates to patients with osteoporosis, Paget's disease, parathyroid diseases, and other disorders of skeletal metabolism. During each infusion, the specific activity of the calcium of plasma was found to increase at a constant rate—a result which allowed calculation of the amount of calcium with which the administered Ca^{45} appeared to mix during the infusion (the miscible calcium). The results of this test were reproducible in any given patient, were similar in normal subjects, and appeared to vary characteristically in several different states of altered skeletal metabolism. Similar results were obtained when strontium^{85} was substituted for calcium^{45}.

ALTHOUGH studies of the distribution and turnover of Ca^{45} in subjects with metabolic bone disease have contributed to our understanding of these states (1–10), such tests have not been used frequently in the clinical evaluation of patients. In part this may be because the usual testing procedures are cumbersome, involving calculation from data collected by measurement of the specific activity of blood, urine and stools during one or more weeks. Consequently, too few studies have been carried out, either in normal subjects or in patients with any given abnormality, to allow valid interpretation of the clinical significance of the results. Not only would a simpler procedure overcome this disadvantage, but would be of value when repeated studies were necessary or when prolonged hospitalization was impractical. Such a relatively simple test was suggested by an earlier investigation of the distribution of intravenously administered calcium gluconate labeled by Ca^{45}. It was found that the administered calcium salt mixed rapidly with part of the exchangeable calcium of the body, and that the calculated miscible pool was similar in normal subjects, but increased in patients whose rate of bone formation was accelerated (11).

This report describes analogous studies in which the rate of infusion of

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Ca^{45} or Sr^{85} of high specific activity was accurately controlled. It was found that during the infusion the activity of plasma increased at a constant rate, that this result was reproducible in repeated tests on a given patient, that the rates observed were similar to each other in normal adults, and that the rates were characteristically altered in patients with different states of abnormal calcium metabolism. These results were used to calculate a quantity of calcium in the body, here called the miscible calcium, with which the isotope appeared to mix during the period of infusion.

CLINICAL MATERIAL AND METHODS

Subjects

Sixty-eight tests were carried out in 42 patients, most of whom were hospitalized during the period of study. Thirteen "normal" patients had no signs or symptoms of skeletal disease or abnormal calcium metabolism, but many had other diseases, such as arteriosclerosis or hypertension.

Methods

Ca^{45} of high specific activity[1] was prepared in isotonic sodium chloride with a final pH of 5, and usually a concentration of 0.5 μc. of Ca^{45} and 3 μg. of stable calcium per ml. In many tests, Sr^{85} (usually 1 μc. of Sr^{85} and 0.1 μg. of stable strontium per ml.) was added to the infusion solution; in other tests, Sr^{85} alone was used. All infusions were administered in the morning to subjects who fasted until the end of the test. The infusion solution was delivered by means of a constant-rate pump[2] at 3.18 ml. per hour for 1.7 to 3.3 hours, during which samples of blood were taken from the opposite arm. When more than one test was performed in the same patient, at least seventy-two hours elapsed between infusions.

The preparation and chemical analysis of samples were carried out by methods previously cited (5, 11). Two different systems for measurement of radioactivity of samples of plasma were used. In some experiments, precipitates of calcium and strontium oxalate were prepared (after addition of enough stable salts of calcium and strontium so that each sample contained 2 mg. of each element) by a modification of the procedure previously used (5, 11). The radioactivity of each sample was then measured by means of a Geiger-Müller tube (when Ca^{45} was present), or by a sodium iodide crystal scintillation detector (when Sr^{85} was present), or both. In other experiments, the radioactivity of plasma was measured directly by use of a scintillation detector (sodium iodide crystal); the calcium thereafter was precipitated as oxalate, and its radioactivity determined by liquid scintillation counting (12). In every case, automatic recording and sample changing equipment was used, and a sufficient number of counts made so that the probable error was less than 1 per cent.

When both Ca^{45} and Sr^{45} were present in the same sample, the counts recorded from the sodium iodide crystal were attributed entirely to Sr^{85}. However, it was necessary to

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[1] Ca^{45}-P-2 was obtained from Oak Ridge National Laboratory on allocation from the U.S. Atomic Energy Commission.

[2] Harvard Apparatus Company, Inc., Dover, Mass.; infusion withdrawal pump 600–900.

correct the counts recorded from either the Geiger-Müller tube or the liquid scintillation detector because of the effect of gamma radiation from Sr^{85}. When B_t and B_s are, respectively, the recorded counting rates from a Geiger-Müller tube or liquid scintillation assembly of a sample T ($Ca^{45}+Sr^{85}$) and a standard of Sr^{85}, and G_t and G_s are the counting rates of the same samples recorded from a sodium iodide crystal, the counting rate from Sr^{85} in sample T of the Geiger-Müller or liquid scintillation assembly $= G_tB_s/G_s$, and the counts from $Ca^{45} = B_t - G_tB_s/G_s$.

Calculations

Activity was expressed as the percentage of Ca^{45} or Sr^{85} administered per gram of calcium. For the purpose of calculation, the quantity of calcium with which the administered isotope appeared to mix during the period of infusion (the miscible calcium) was arbitrarily treated as a single compartment of uniform specific activity and not subject to appreciably rapid turnover.[3] It follows, from the assumption that specific activity of miscible calcium and plasma are the same, that the rate of change of specific activity of miscible calcium is given by the rate of change of specific activity in plasma. Accordingly, when Ca^{45} is infused, $S = Ca^{45}/Ca^{40}$, where S is specific activity, Ca^{45} is the percentage of the dose administered, and Ca^{40} is grams of miscible calcium. Therefore, during the infusion, $dS/dt = (Ca^{40})^{-1}dCa^{45}/dt$, and miscible calcium $= (dCa^{45}/dt)/(dS/dt)$. The rate of change of specific activity (dS/dt) of miscible calcium was obtained from the calculated regression (method of least squares) of the measured specific activity in plasma upon time after start of infusion (for practical purposes, the rate is given by the slope of the straight line which best fits the data when plotted as in Figures 1 and 2). Samples taken during the first twenty minutes of infusions were not used for calculation of slope. No correction was necessary for the small amounts of isotope excreted in urine during the infusion (see subsequent data).

When Sr^{85} was used, similar calculations were made, based on the relationship, miscible calcium $= (dSr^{85}/dt)/(dS'/dt)$, where dSr^{85}/dt is the rate of infusion and S′ is the relative specific activity (per cent of the dose of Sr^{85} per Gm. calcium of plasma). As an example of the calculation, in the study illustrated by the top curve of Figure 1, the infusion was given for 3.0 hours (rate, 33.3 per cent per hour) and the slope of the calculated regression line was 30.0 per cent of the dose per Gm. calcium per hour. The miscible calcium (Gm.) was $33.3/30.0 = 1.11$.

Probability estimations were based on "Student's" *t* distribution and were made by standard statistical methods (13).

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[3] More explicitly stated, dS/dt is a function, not only of the rate of infusion and size of pool, but of the rate of turnover (assumed to be exponential) of the pool. The assumption that turnover of miscible calcium is slow relative to rate of infusion is approximately satisfied by the observed linear relationship between specific activity and time of infusion. If turnover were rapid or if the infusion were continued for a long enough period, the rate of change of specific activity would fall progressively (because ions of Ca^{45} would be replaced by non-radioactive ions from some other site). The second assumption, that miscible calcium is a pool of uniform specific activity, is made for purposes of calculation only. Because of the complex nature of the reactions between the calcium ions of extracellular fluid and at the surface of bone (which presumably occur at a large number of different rates depending upon anatomic location and physical state of bone surface) any attempt to define a single pool is probably arbitrary.

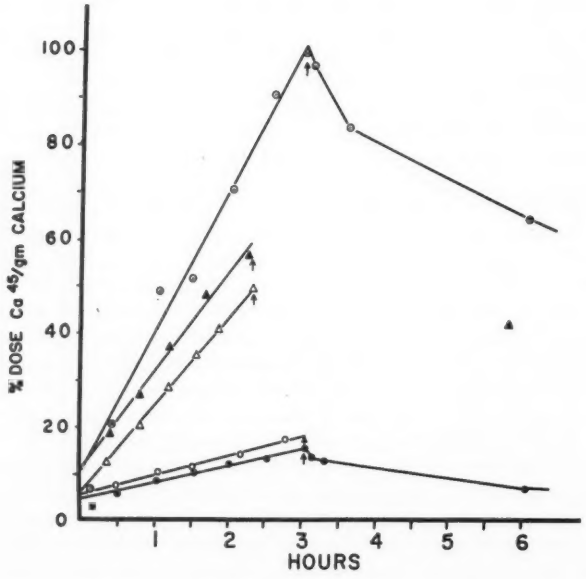

FIG. 1. Comparison of changes in specific activity of plasma calcium during and after five continuous infusions of Ca^{45} into 3 patients with different abnormalities of calcium metabolism. The end of each infusion is indicated by an arrow. Diagnoses were as follows: ⊙, hypoparathyroidism (Patient B.R.); ▲ and △, osteoporosis (Patient V.M.); ● and ○, Paget's disease (Patient W.T.).

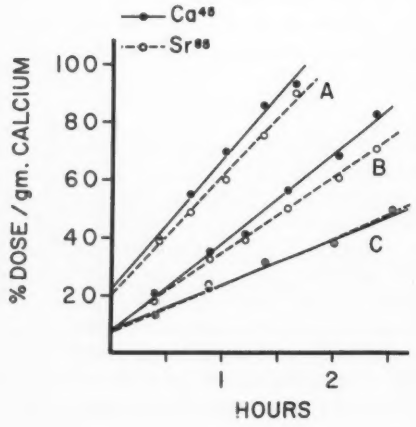

FIG. 2. Comparison of results when Ca^{45} and Sr^{85} were given simultaneously. Three studies are shown. The regression lines were calculated by the method of least squares, and the possibility that the slopes (Ca^{45} versus Sr^{85}) differed more than could be accounted for because of variation of the data was examined by use of the "t" test. In two studies, the slopes calculated from Ca^{45} and Sr^{85} were not significantly different (for study C, $P \sim .5$; for study A, $.10 < P < .15$). Variation between slopes, although slight, was significant in study B ($P < .01$).

RESULTS

Figure 1 shows the results of five infusions of Ca^{45} given to 3 patients (B.R., V.M. and W.T.) with various abnormalities of calcium metabolism. Four general features of the effects of such infusions on the specific activity of plasma calcium are illustrated. First, in each case the specific activities, when plotted on Cartesian ordinates against time after the start of infusion, adequately described a straight line. This linear relationship was apparent within five minutes in some tests but only after the first twenty minutes of infusion in others. Second, the regression lines often did not pass through zero. Third, when infusions were given to the same subject for similar periods, the observed slopes also were similar, but when tests were carried out in subjects with different states such as osteoporosis and Paget's disease, markedly different slopes were observed. Fourth, as soon as the infusions were discontinued, the specific activity fell, at first rapidly and then more slowly.

The precision of any given estimate of miscible calcium is indicated by the standard error of the slope (S_b) from which it was calculated. (Because the rate of infusion is assumed to be constant, the slope and the miscible calcium have the same fractional variance.) The standard deviation of S_b in 38 tests so studied was 3.7 per cent of the slope.

The reproducibility of the measurement of miscible calcium in any given patient was estimated in 23 tests on 10 subjects (tests a, b, c, or d in Tables 1 through 4). The standard deviation from the mean value of these tests was 5.9 per cent. (Because the second or third infusion had been given in Patients M.D., L.L., B.T. and W.T. only after one or more months of

TABLE 1. MISCIBLE CALCIUM OF 13 SUBJECTS WITH NORMAL SKELETAL AND CALCIUM METABOLISM

Patient	Test	Sex & age	Miscible calcium (mg./Kg.)
B.F.	Ca^{45}	F 69	33.5
O.H.	Ca^{45}	M 67	33.5
R.C.	Sr^{85}	M 61	29.3
R.H.	Ca^{45}	M 59	36.9
C.D.	Sr^{85}	M 53	30.7
L.B.	Sr^{85}	F 49	26.0
"	Ca^{45}		29.4
I.W.	Ca^{45}	M 49	35.9
J.F.	Ca^{45}	M 48	27.5
S.J.	a—Ca^{45}	F 42	29.3
"	b—Ca^{45}		29.3
M.S.	Ca^{45}	F 41	27.5
R.T.	Ca^{45}	M 36	26.2
M.F.	Sr^{85}	F 33	27.5
O.M.	Sr^{85}	M 27	30.3
			Mean ± S.D. = 30.2 ± 3.3

hospitalization, when altered diet, activity or other condition may have affected the skeletal metabolism, the true duplicate variability may be somewhat less.) This result indicates that the variation between tests on the same subject was comparable to the uncertainty of the individual calculations.

Ca^{45} and Sr^{85} were given simultaneously in 36 tests. Three typical studies are plotted in Figure 2, which illustrates the similarity of results obtained

TABLE 2. MISCIBLE CALCIUM OF 14 PATIENTS WITH OSTEOPOROSIS

Type of osteoporosis	Patient	Test	Sex & age	Miscible calcium (mg./Kg.)
Senile	M.C.	Ca^{45}	M 73	56.4
	K.M.	Sr^{85}	M 73	38.5
	C.W.	Ca^{45}	M 63	48.8
	B.B.	Ca^{45}	M 60	48.2
Postmenopausal	E.O.	Ca^{45}	F 74	22.8
	M.G.*	Ca^{45}	F 58	40.1
	M.D.	a—Ca^{45}	F 56	45.8
	"	b—Ca^{45}		34.0
	V.M.*	a—Ca^{45}	F 52	30.7
	"	b—Ca^{45}		35.8
	"	c—Ca^{45}		34.1
	V.K.	Ca^{45}	F 52	35.2
	L.L.*	a—Ca^{45}	F 50	30.9
	"	b—Ca^{45}		37.3
	"	c—Ca^{45}		39.1
	"	d—Ca^{45}		32.0
	L.M.	Ca^{45}	F 42	31.0
Steroid-induced	T.C.	a—Ca^{45}	F 54	24.1
	"	b—Ca^{45}		21.7
Idiopathic	R.W.	Ca^{45}	M 55	24.3
	R.D.	Ca^{45}	F 28	25.7

* There was x-ray evidence of skeletal decalcification, and symptoms which suggested postmenopausal osteoporosis, but no vertebral compression fractures.

by use of these isotopes. When slopes calculated from changes in relative activity were expressed, for each test, as the percentage of $\frac{1}{2}$ (slope from Ca^{45}+slope from Sr^{85}), their mean was 99.7 and standard deviation 7.3; this value did not differ significantly from those similarly calculated for slopes from Ca^{45} ($P > .4$). Therefore, although the slopes derived from Ca^{45} and Sr^{85} occasionally were significantly different in respect to S_b, the slopes calculated from the use of one isotope were not consistently greater or less than the other, and the variance was only slightly greater than that of duplicate tests on any given patient.

The amount of isotope excreted in the urine during the period of infusion was studied in 14 tests when Ca^{45} was given, and in 7 tests when Sr^{85} was given. Although specific activity was high, the total amount of calcium ex-

creted during the three hours of infusion was small. Consequently the total amount of isotope excreted was negligible (0.33 ± 0.25 per cent of the dose of Ca^{45}; 1.27 ± 0.93 per cent of the dose of Sr^{85}), being most in patients with osteoporosis and least in patients with osteomalacia and Paget's disease.

Because both the duration of infusion and the quantity of miscible calcium affected the calculated slope, the different studies were better compared on the basis of the derived data (miscible calcium). In Tables 1 through 4 the quantities of miscible calcium in all patients studied are listed. Calculations were made from changes in specific activity, except when only Sr^{85} was given. In Table 1, the miscible calcium of 13 patients without skeletal disease is shown (30.2 ± 3.3 mg. per Kg. of body weight).

Results in 14 patients with osteoporosis are listed in Table 2. Except in 3 patients, skeletal demineralization was confirmed by the presence of a compression fracture of a vertebra; in every case the clinical and x-ray findings otherwise supported the diagnosis. Four patients with senile osteoporosis had definitely increased amounts of miscible calcium; according to the history, the dietary intake had been adequate in 3; one (C.W.) had voluntarily restricted his intake mainly to carbohydrates for eight months. The 7 patients with postmenopausal osteoporosis had either normal or slightly elevated values for miscible calcium. In the only patient with osteoporosis secondary to corticosteroid therapy (for rheumatoid arthritis), the value for miscible calcium was low. Miscible calcium was normal in 2 subjects with idiopathic osteoporosis.

Studies on 9 patients with other metabolic bone diseases are shown in Table 3. Miscible calcium was low in 2 untreated hypoparathyroid subjects, but increased when they were treated with vitamin D_2 and a diet relatively deficient in phosphate supplemented by calcium gluconate. The miscible calcium was elevated in 2 of the hyperparathyroid patients, but at the upper limits of normal in the third (none of them had osteitis fibrosa generalisata). In 2 hyperthyroid subjects, the miscible calcium was unremarkable in one but at the upper limits of normal in the other. In the single patient with very severe osteomalacia secondary to nontropical sprue, the amount of miscible calcium was markedly increased. When she was treated by a gluten-free diet there was rapid symptomatic and physical improvement; after two months, the miscible calcium had returned to almost normal values.

The results in 8 patients with various skeletal diseases are listed in Table 4. The miscible calcium was normal or moderately increased in a subject with fibrous dysplasia, and in 3 subjects with inactive Paget's disease. Each (Patients H.H., J.D., A.S. and L.H.) had localized lesions which had been relatively inactive for a decade, minimal evidence of osteolysis, and serum alkaline phosphatase activity of less than 40 King-Armstrong units per

TABLE 3. MISCIBLE CALCIUM OF 9 PATIENTS WITH METABOLIC BONE DISEASES OTHER THAN OSTEOPOROSIS

Diagnosis	Patient	Test	Sex & age	Miscible calcium (mg./Kg.)
Hypoparathyroidism				
treated (2 yrs.)	K.S.	Ca^{45}	F 64	20.8
untreated	B.R.	Ca^{45}	M 50	16.3
treated (8 wks.)	"	Ca^{45}		19.7
untreated	Z.T.	Sr^{85}	F 30	21.8
treated (8 wks.)	"	Sr^{85}		28.6
Hyperparathyroidism	P.B.	Ca^{45}	M 70	42.0
	W.D.	Ca^{45}	M 69	64.0
	A.J.	a—Ca^{45}	M 63	37.0
	"	b—Ca^{45}		37.0
Hyperthyroidism	A.B.	Ca^{45}	F 83	37.0
	W.T.	Sr^{85}	F 48	28.5
Osteomalacia	B.T.	a—Ca^{45}	F 61	70.7
untreated	"	b—Ca^{45}		59.8
treated	"	Ca^{45}		39.8

100 ml. In contrast, the miscible calcium was markedly elevated (84 to 176 mg. per Kg.) in 3 patients with widespread, rapidly progressive Paget's disease and serum alkaline phosphatase activity of 74 to 160 King-Armstrong units per 100 ml. In the single patient with multiple myeloma, whose plasma calcium concentration was 15.3 mg. per 100 ml., the miscible calcium was markedly abnormal (96 mg. per Kg.).

The localized nature of the skeletal lesions in 2 of the patients listed in Table 4 (L.H. and H.H.) made it possible to compare the uptakes in nor-

TABLE 4. MISCIBLE CALCIUM OF 8 PATIENTS WITH VARIOUS DISEASES AFFECTING THE SKELETON

Diagnosis	Patient	Test	Sex & age	Miscible calcium (mg./Kg.)
Paget's disease				
inactive	J.D.	Ca^{45}	M 73	51.0
inactive	H.H.	Ca^{45}	F 68	40.6
inactive	A.S.	Ca^{45}	M 65	35.6
progressive	I.S.	a—Ca^{45}	M 79	176.0
progressive	"	b—Ca^{45}		154.0
progressive	H.R.	a—Ca^{45}	M 62	84.4
progressive	"	b—Ca^{45}		119.0
progressive	W.T.	a—Ca^{45}	M 62	143.0
progressive	"	b—Ca^{45}		124.0
Fibrous dysplasia	L.H.	Sr^{85}	M 48	38.5
Multiple myeloma (hypercalemic)	I.T.	Ca^{45}	F 34	96.0

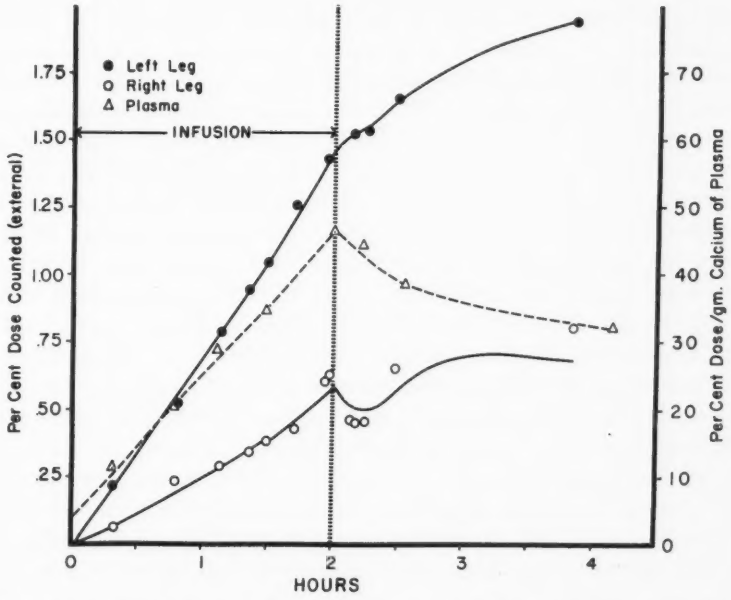

FIG. 3. Comparison of rates of accumulation of Sr^{85} in the right tibia (normal) and left tibia (osteitis of Paget's disease) during an infusion of 6 μc. of Sr^{85} per hour. The rate of change of relative specific activity of calcium in plasma also is plotted.

mal and abnormal bone by external counting during administration of Sr^{85}. The results of both studies were similar and, as illustrated in Figure 3, showed markedly increased uptake by the abnormal bone. The same accelerated uptake was indicated by the progressively increased accumulation of Sr^{85} after the infusion was discontinued, when the relative specific activity of calcium of plasma was falling.

DISCUSSION

The altered rates of change of specific activity during tests of subjects with skeletal disease can best be explained as the result of either accelerated or subnormal uptake of isotope by bone. This was shown directly by external counting, during an infusion of Sr^{85}, over diseased and normal bone in a patient with Paget's disease (Fig. 4); the rate of increase of radioactivity was three times greater over the osteitic bone than over the normal bone. Because the rate of bone formation is too slow (about 20 mg. per hour) to have a significant effect during such short studies, the observed uptake must have been largely the result of accelerated exchange between

the ions of extracellular fluid (E.C.F.) and the non-radioactive calcium ions at or near the surface of bone. (It has been shown (2) that about 19 per cent of calcium ions of plasma mix per minute with those of E.C.F., a process so rapid in comparison to the time scale of this test that, for practical purposes, plasma and other E.C.F. may be assumed to have the same specific activity.) Accelerated exchange between the calcium of E.C.F. and the calcium of bone could result from: 1) increased surface area of bone crystals exposed to E.C.F., 2) accelerated exchange over any given surface area, or 3) increased blood flow to bone. Of these processes, the first only affects the total exchangeable calcium, the latter two affecting the rate but not the ultimate extent of distribution of calcium ions. Therefore results of tests, such as the continuous Ca^{45} infusion test, which principally reflect changes in the distribution of calcium in the first few minutes after its administration, do not necessarily furnish the same information as do measurements of the total exchangeable calcium.

Although the present studies do not show which processes operate in any of the conditions studied, they do indicate when the rapid exchange of calcium ions between E.C.F. and bone is more (or less) extensive than normal. An expression of this result is given by calculation of the miscible

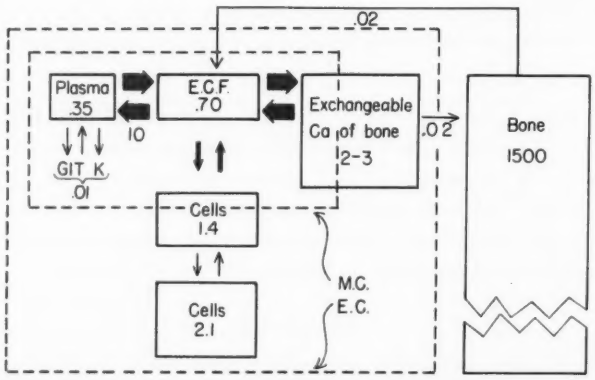

FIG. 4. A model consistent with the distribution of calcium in the body. The quantities (Gm.) and rates (Gm. per hour) are extrapolated from studies carried out, for the most part, in other mammals, and are meant to indicate orders of magnitude rather than actual mass or flux for a 70-Kg. human. Where no applicable data exist, the probable relative magnitude of the rate is indicated by the thickness of the arrow. The model is greatly simplified; for instance, all cells of the body are treated as if their calcium metabolism were identical, and the exchangeable calcium of bone is treated as a single homogeneous compartment. GIT = gastro-intestinal tract; K = kidney; E.C.F. = extracellular fluid other than plasma; E.C. and M.C. indicate the probable extent of the total exchangeable calcium and miscible calcium.

calcium, or the pool with which the administered isotope appeared to mix during the infusion. Because this calculation rests upon assumptions which cannot be fully justified, the miscible calcium must be looked upon as an empirical pool. It is presumed to consist of the calcium of E.C.F. and the most readily available calcium of bone. In Figure 4 it is contrasted with the total exchangeable calcium.

The relatively small variation between results of tests in normal subjects (Table 1) and of duplicate tests in the same subject, suggests the possible usefulness of this procedure in metabolic study. Preliminary results are indicated in the response to treatment of the patient with nontropical sprue and the 2 hypoparathyroid subjects.

In addition, we have studied the variation of miscible calcium in several metabolic and skeletal diseases. The changes in patients with Paget's disease, hyperparathyroidism and hypoparathyroidism seem to indicate a correlation between miscible calcium and the rate of bone formation similar to that previously suggested for the rate of bone formation and total exchangeable calcium (6, 7). The latter correlation may result because, when bone formation is accelerated, an increased amount of newly formed, as yet incompletely calcified bone is present. It is thought that exchange reactions with the ions of E.C.F. occur over a relatively large surface area of newly formed bone because the crystals are less densely packed than in older, fully calcified bone (14). Such theoretical considerations are supported by *in vivo* and *in vitro* studies which show the uptake of Ca^{45} by exchange to be most intense in newly formed osteones (15, 16). This theory affords the most reasonable explanation of the observed correlation between the rate of bone formation and total exchangeable calcium (6, 7), and probably largely explains the similar changes in miscible calcium in the patients with Paget's disease and with abnormal parathyroid function in the present study. The abnormally large amount of miscible calcium found in the patient with osteomalacia may also have been the result of exchange reactions between the calcium of E.C.F. and a larger-than-normal quantity of poorly mineralized bone. However, it is possible that other factors, such as increased skeletal circulation or changes of pH resulting in altered solubility of bone also affect the miscible calcium.

The most striking difference between the results of this and past studies was related to osteoporosis. In past studies (6, 7, 9), the rate of bone formation and total exchangeable calcium of osteoporotic subjects was found to be normal.[4] In contrast, our results show that miscible calcium is signifi-

[4] In one study (9), several measurements on osteoporotic subjects indicated the possibility of an increased rate of bone formation (but normal total exchangeable calcium). According to our calculation ("*t*" test) of this author's data, these results were not significantly different from measurements on his normal subjects.

cantly increased in a large percentage of patients, particularly those with senile osteoporosis.[5] The reason is not clear, nor is it yet known whether changes in miscible calcium can be correlated with the clinical status or with the calcium balance of these osteoporotic subjects. We tentatively interpret these results as indicating a physical change at the surface of some part of bone exposed to E.C.F., which results in accelerated exchange. Whether this could be related to the presence of increased osteoclastic activity, the abnormal presence of poorly mineralized bone, or to some other process cannot be determined at present. The effect of different levels of calcium and phosphorus intake and of treatment of osteoporotic subjects is now under study.

Similar, but not always identical results were obtained when Sr^{85} was given instead of Ca^{45}. This finding has some practical significance, because (as with Ca^{47}) the measurement of Sr^{85} activity in serum can be carried out without extensive sample preparation, and because the radiation dose to bone from Sr^{85} is much smaller than from Ca^{45}.

Acknowledgments

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ADRENAL ESTROGENS. I. CONVERSION OF 4-C^{14}-CORTISONE ACETATE TO 11-OXYGENATED ESTROGENS IN WOMEN

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ABSTRACT

The bioconversion of adrenal corticosteroids to phenolic estrogens was investigated. After the administration of 4-C^{14}-cortisone acetate to a castrated and adrenalectomized woman with breast cancer, 11β-hydroxyestrone and 11β-hydroxyestradiol were isolated from the urine. The radiochemical purity and identity of the C^{14}-labeled conversion products were ascertained by paper chromatography, counter-current distribution, and carrier crystallization. After administration of 4-C^{14}-cortisol to the same patient, however, this conversion did not occur.

IN THE biosynthesis of estrogens, the adrenal cortex contributes part of the total estrogen pool. This bas been conclusively demonstrated by the following observations: the decrease or disappearance of urinary estrogen following adrenalectomy in women with mammary cancer (1); the increase of estrogen excretion in castrated women following stimulation by adrenocorticotropic hormone (ACTH) and the absence of such an increase in castrated and adrenalectomized patients (2); the increase of estrogen excretion in patients with adrenocortical hyperplasia and feminizing adrenocortical tumors (3); and the isolation of estrone from bovine adrenals (4). All these observations are evidence that the adrenal cortex is a source of extragonadal estrogens.

The isolation and identification of urinary 17-ketosteroids following the administration of 17-hydroxycorticosteroids led Dorfman and his associates (5–6) to implicate the adrenal cortex as a contributor to the total 17-ketosteroid pool. The 17-ketosteroids of adrenal origin were identified by the presence of an oxygen function at C-11.

This paper describes the isolation and identification of 11β-hydroxyestrone[1] and 11β-hydroxyestradiol from the urine of a castrated and adrenalectomized woman with metastatic breast cancer following the administration of a tracer dose of 4-C^{14}-cortisone acetate.

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[1] Standard 11β-hydroxyestrone was generously supplied by Dr. J. A. Hogg who, with his associates, first successfully synthesized both 11β-hydroxyestrone and 11β-hydroxyestradiol.

MATERIALS AND METHODS

Determination of radioactivity. All samples for analysis of radioactivity following counter-current distributions were plated on counting planchets and counted in a windowless gas flow counter operated in the Geiger region. The radioactivity found was corrected for background and self-absorption, and expressed as counts per minute per tube (cpm/tube).

Determination of estrogens. Standard estrogens used as carriers for the radioactivity were analyzed fluorometrically. Conditions for the formation of fluorescence are those described by Bates and Cohen (7). Intensities of fluorescence were measured by a Farand photofluorometer with 4358 A and 4800 A Baird-Atomic interference filters. The "fluorescent extinction coefficient" of 11β-hydroxyestradiol is similar to that of estrone, whereas that of 11β-hydroxyestrone is approximately 20 per cent of that of estrone.

11-oxygenated estrogens. 11β-Hydroxyestradiol was prepared in our laboratory by sodium borohydride reduction of 11β-hydroxyestrone as follows: 310 mg. of 11β-hydroxyestrone was dissolved in 50 ml. of 1 per cent aqueous sodium hydroxide at room temperature. To the alkaline solution was added 150 mg. of sodium borohydride; the mixture was stirred by means of a magnetic stirrer for four hours. At the end of the reaction period, the mixture was poured into 500 ml. of 1 per cent hydrochloric acid solution. When the evolution of gas ceased, tiny crystals began to appear. The aqueous mixture was allowed to stand at 0°C. for twelve hours; then the crystalline residue was collected. The filter cake was washed copiously with water till the washing was free of acid. The residue weighed approximately 170 mg. A 10-mg. sample of the crude material was purified by counter-current distribution, and then was recrystallized from aqueous ethanol. The infrared spectrum and the chromatographic mobilities of the reduced material were identical with those of the analytical sample of 11β-hydroxyestradiol as supplied to us by Hogg.

Radioactive urinary extract. A tracer dose (10 μc.) of 4-C^{14}-cortisone acetate was given orally to a patient who had undergone bilateral oophorectomy and adrenalectomy for metastatic cancer of the breast. The study was made five months after the adrenalectomy, when the patient was receiving a maintenance dosage of 50 mg. of cortisone acetate by mouth daily. Three 24-hour urine samples were collected.

The urine samples were pooled, and the steroids were hydrolyzed with Ketodase[2] (300 units per ml. of urine) at pH 4.5 for forty-eight hours at 37°C. The hydrolysate was subjected to extraction with $\frac{1}{4}$ volume of ether in a continuous extractor for twenty-four hours. After separating the ether phase, the aqueous phase was adjusted to pH 1 with concentrated sulfuric acid. The pH 1 mixture was incubated and extraction was carried out as before. The ether extracts were combined and fractionated into neutral, phenolic, and acidic fractions by means of the procedure described by Engel (8). The phenolic fraction was further separated by counter-current distributions with systems adapted for estrogen separation by West and associates (9). The neutral fraction was submitted to gradient elution chromatography (10) for the analysis of 17-ketosteroids. A flow diagram of the method of fractionation of the urinary extract is summarized in Figure 1.

Counter-current distributions. The basic technique of the counter-current distribution and the method of calculating partition coefficient and theoretical curves were developed

[2] Purified beef liver glucuronidase (Ketodase), manufactured by Warner Laboratories, New York, N. Y.

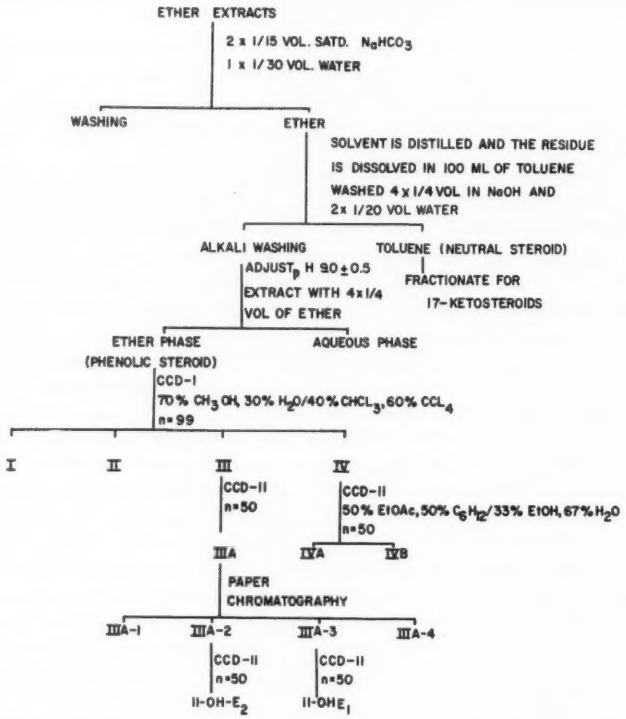

FIG. 1. A flow diagram of the method of fractionation of the urinary extract.

by Craig and Craig (11). A 100-cell, all-glass, automatic machine was employed for this study. The total solvent capacity for a single cell was 20 ml. Equal volumes of upper and lower phases of a solvent system were used, and the upper layer was the moving phase.

Partition coefficients of 11β-hydroxyestrone and 11β-hydroxyestradiol were determined by counter-current distribution of the standard with 50 transfers for each respective system. It was found, as the result of several separate distributions, that the position of the peak tubes may vary from 0 to 3 tubes. This variation may be attributed to the variation of temperature and other factors affecting the equilibrium of the solute between the phases of the system. Partition coefficients of 11β-hydroxyestrone and 11β-hydroxyestradiol as well as those of the classic estrogens are summarized in Table 1.

Paper chromatography. Following the redistribution, the radioactive metabolites were further separated by paper chromatography. Paper chromatography was carried out by a slight modification of the method described by Bush (12). Whatman #3 paper was used, and the chromatograms were developed with benzene as the mobile phase and 65 per cent methanol as the stationary phase at room temperature for two hours. Standards were run parallel to the unknown for each chromatogram, and the mobilities were expressed in centimeters as measured from the origin to the center of the spot. The chromatogram containing the standards was developed by spraying with Folin reagent

TABLE 1. SOLVENT SYSTEMS USED IN COUNTER-CURRENT DISTRIBUTION AND IN DETERMINING PARTITION COEFFICIENTS FOR VARIOUS ESTROGENS

Solvent system No.	Solvent	K (partition coefficient)				
		Estrone*	Estradiol-17β*	Estriol*	11β-OH-estrone	11β-OH-estradiol
I	70% CH_3OH—40% $CHCl_3$, 60% CCl_4	0.21	1.02	5.45	1.14	3.90
II	50% EtOAc, 50% c-C_6H_{12}-33% EtOH	—	—	1.20	2.84	1.27

* These values were previously reported by West *et al.* (10).

and dipping in an atmosphere of ammonia till the blue spots became visible. The radioactive spots were determined by cutting the chromatogram into unit areas per centimeter from the origin, and eluting them with ethanol; an aliquot of eluate was plated on the planchets and counted. Radioactivities were expressed as counts per minute per area per centimeter (cpm/a/cm.).

Counter-current distribution of radioactive metabolites with carrier. To the eluates from radioactive spots with mobilities corresponding to the standards was added 2 mg. of carrier, and the solute was distributed in system II with 50 transfers. After distribution was completed, every other tube was analyzed for radioactivity and carrier concentration. Any nonhomogeneity of the radioactive metabolite and the carrier would be apparent, since in such a case the peak tubes found by the two different methods of analysis do not coincide. Two different theoretical curves would be needed to fit the two sets of data. On the other hand, if the peak tubes coincide, then the chemical identity of the radioactive compound and the carrier can be ascertained.

Carrier crystallization. For further identification of the isolated radioactive metabolites, an isotopic dilution technique was employed. Material from each carrier counter-current distribution was pooled, and 10–20 mg. of recrystallized carrier was added. The identification was considered complete when there was no change in specific activity following successive recrystallizations.

RESULTS

Preliminary distribution of phenolic extract

The preliminary distribution of phenolic extract (144,350 cpm; 1.05 per cent of total radioactivity administered) in system I with 99 transfers showed four bands. Band III (tubes 51–69; 58,560 cpm) and band IV (tubes 70–96; 76,620 cpm) contained a majority of the counts. The less polar bands (I and II) contained no immediately recognizable metabolites, and hence were saved for future analysis. The present effort was concentrated mainly on bands III and IV.

Redistribution of bands III and IV

Redistribution of band III in system II with 50 transfers revealed a single band (III-A) between tubes #21–40, with 47,652 cpm. Redistribution of band IV revealed two bands at tubes #2–20 (IV-A) and #41–50 (IV-B), with 56,500 cpm and 10,464 cpm repsectively.

Counter-current distribution of III-A, IV-A and IV-B between toluene and 1 normal NaOH

Since the total radioactivity isolated was very small compared with the total dose of the tracer, the radiochemical purity was ascertained by 8 counter-current transfers between toluene and 1 *N* sodium hydroxide solution, as described by Baggett *et al.* (13). The distribution of the radioactivity is shown in Figure 2. Most of the radioactivity was concentrated in tube #8, indicating the presence of a C^{14}-labeled phenolic compound rather than a neutral labeled contaminant partitioned into the phenolic fraction. Distribution of 11β-hydroxyestrone in this system also showed

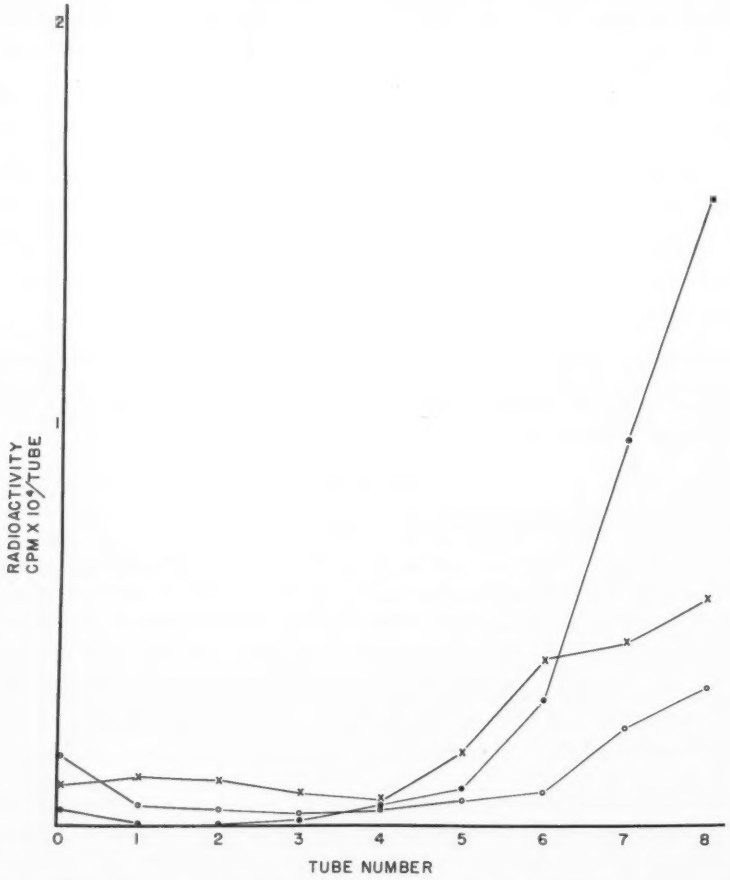

FIG. 2. Counter-current distribution of the phenolic fractions in toluene and 1 *N* sodium hydroxide. ×—× = III-A; ●—● = IV-A; ○—○ = IV-B.

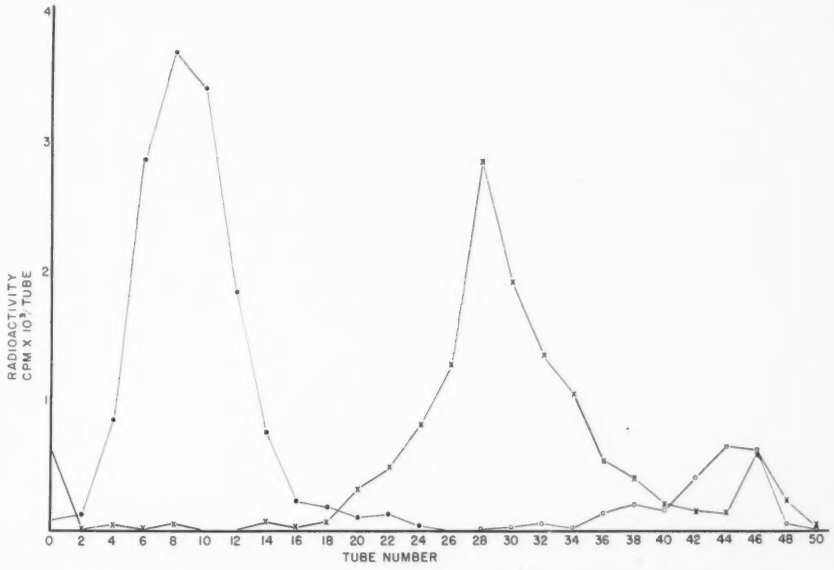

FIG. 3. Counter-current distribution of phenolic fractions in solvent system II.
×—× = III-A; ●—● = IV-A; ○—○ = IV-B.

concentration of fluorescent material in tube #8. Radioactivity between tube #5 and tube #8 was pooled and again distributed in system II.

Redistribution of III-A, IV-A and IV-B

Partition between toluene and 1 *N* sodium hydroxide solution removed a considerable amount of the neutral urinary chromogens. The redistribution curve is shown in Figure 3. Because of a lack of proper standards, the peaks of IV-A and IV-B still cannot be identified. The peak tube of fraction III-A showed a partition coefficient of 1.27, and the skewed contour on the descending limb at tube #34 exhibited a shoulder with a partition coefficient of 2.26. Since these partition coefficients were similar to those of 11β-hydroxyestrone and 11β-hydroxyestradiol within the limits of experimental error, the whole fraction was pooled and separated by paper chromatography.

Paper chromatography of III-A

Paper chromatography of III-A showed peaks at 8 cm. and 15 cm., which corresponded in mobility to those of 11β-hydroxyestradiol and 11β-hydroxyestrone, respectively (Fig. 4). It is evident that the natures of these radioactive metabolites were similar to those of the standards.

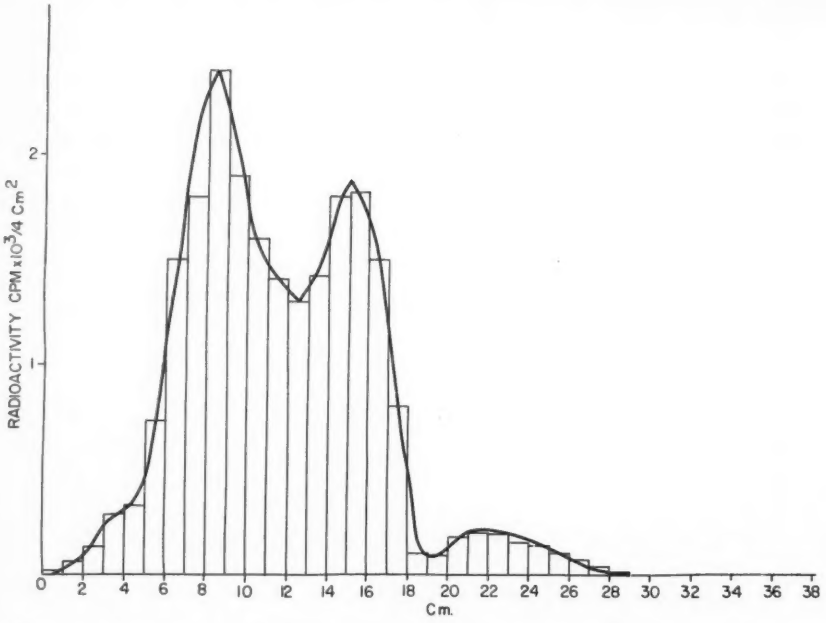

FIG. 4. Paper chromatogram of phenolic fraction III-A, following counter-current distribution in solvent system II. Peaks at 9 cm. and 15 cm. corresponded in running rate with that of standard 11β-hydroxyestradiol and 11β-hydroxyestrone respectively.

Counter-current distribution with carrier

The radioactivities of 5–12 cm. and 13–19 cm. were pooled, and 2 mg. of each of the 11-oxygenated standards were added as the carrier and distributed with 50 transfers in system II. Both radioactivity assay and fluorometric estimation of the carrier closely fitted the calculated theoretical curve (Figs. 5 and 6). From both analyses, the peak of radioactivity and carrier concentration occurred in the same tube. The tubes under each peak were pooled and submitted to carrier crystallization. The results of the carrier crystallizations are summarized in Table 2.

Gradient elution of the neutral fraction

Mixed gradient elution chromatography of the radioactive neutral fraction and 1 mg. each of standard dehydroisoandrosterone, androsterone, etiocholanolone, and 11-ketoetiocholanolone were carried out. After the elution, the standard 17-ketosteroids were analyzed colorimetrically by the Zimmermann method and the unknown material was analyzed by

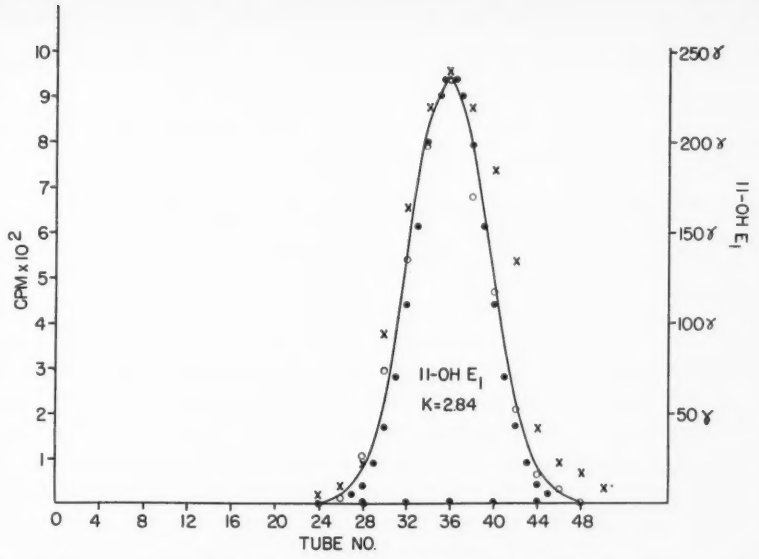

FIG. 5. Counter-current distribution of the radioactive material eluted from the chromatogram with added standard 11β-hydroxyestrone as carrier. × = fluorometric analysis; ○ = radioactivity assay; ● = calculated value (K = 2.84).

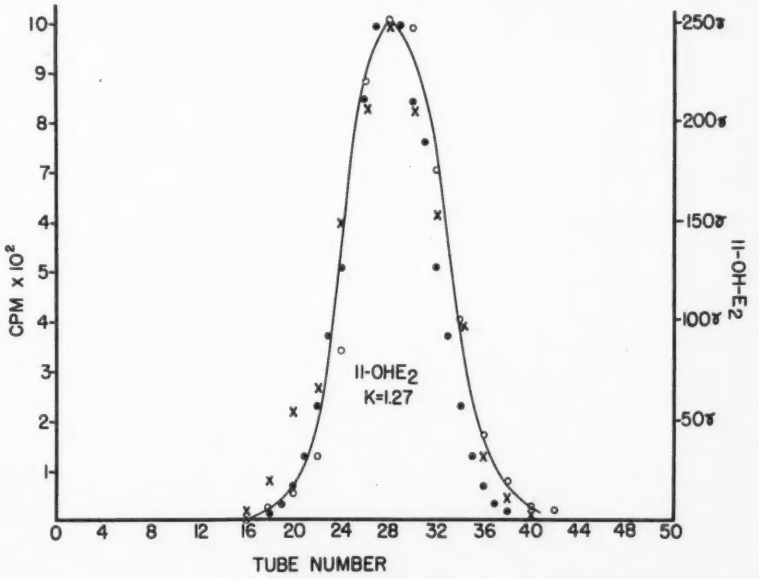

FIG. 6. Counter-current distribution of radioactive material eluted from the chromatogram with added standard 11β-hydroxyestradiol as carrier. × = fluorometric analysis; ○ = radioactivity assay; ● = calculated value (K = 1.27).

TABLE 2. URINARY RADIOACTIVE 11β-HYDROXYESTRONE AND 11β-HYDROXYESTRADIOL AS DETERMINED BY CARRIER RECRYSTALLIZATION

Sample	Carrier	Solvent	Specific activity (cpm/mg.)		
			Before	1st recryst.	2nd recryst.
CCD Tubes #28–44	11β-Hydroxyestrone (28 mg.)	Methanol	115	107	102
CCD Tubes #20–36	11β-Hydroxyestradiol (17 mg.)	Methanol	287	184	198

radioactivity assay. Two radioactive peaks were found (Fig. 7), and they represented more than 85 per cent of the total radioactivity present in the neutral fraction. These metabolites were derived from the conversion of the administered tracer cortisone. Their chemical identities, however, have not been ascertained up to the present. Since cortisone and cortisone acetate were not eluted when subjected to the identical experimental conditions, the nature of these neutral metabolites warrants further study.

Experiment with 4-C^{14}-cortisol acetate

A tracer dose of 4-C^{14}-cortisol acetate was given orally to the same patient. After use of the same methods for isolation and fractionation of compounds in the urinary extract, 11-oxygenated estrogens could not be

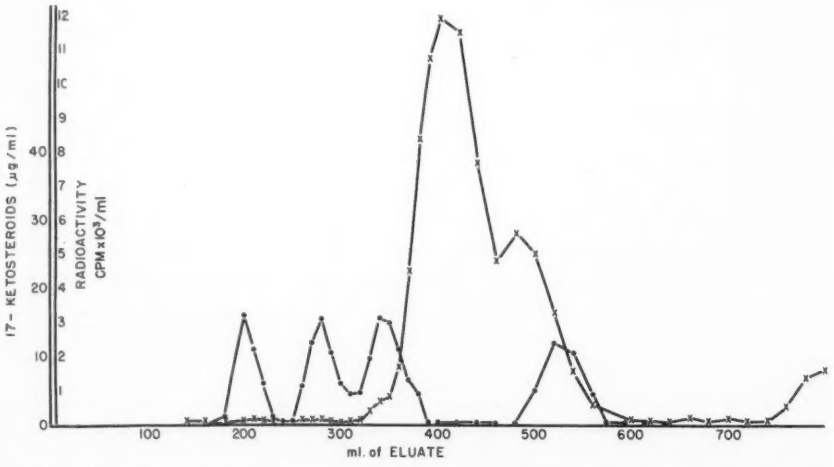

FIG. 7. Gradient elution chromatography of the neutral fraction (×—×) and 4 mg. of mixed 17-ketosteroids standards (●—●). Peaks for the standards are, from left to right, dehydroepiandrosterone, androsterone, etiocholanolone, and 11-ketoetiocholanolone.

detected. Counter-current distribution in system II showed that all the radioactivity was concentrated between tubes #6 and #28. Paper chromatography of this peak revealed the same mobility as that of IV-A.

DISCUSSION

The data presented in this paper appear to provide conclusive evidence that cortisone can be converted to 11-oxygenated estrogens. As far as our present knowledge is concerned, the methods selected for hydrolysis, isolation, radiochemical purification and identification are not likely to cause chemical alteration of the converted metabolites under the conditions used. The purified and identified conversion product is approximately 7 per cent of the phenolic fraction, or 0.07 per cent of the total administered radioactivity. Since the magnitude of conversion is of a very small order, it seems that the major components of adrenal estrogens may be the conversion products of other corticoids, such as 17-hydroxy-11-deoxycorticosteroids or 17-hydroxyprogesterone. Recent studies have indicated that 17-hydroxyprogesterone plays a major role in the biogenesis of steroid hormones.

The present study does not indicate the site of conversion nor the mechanism involved. It may be assumed that mammalian tissues, whether normal or cancerous, possess the necessary enzyme systems which will elicit profound molecular alterations of a C_{21} steroid to a benzenoid C_{18} estrogen. In view of the significance associated with the findings of the present study, the site of conversion and the mechanism involved are worthy of further investigation.

Surprisingly, after the administration of labeled hydrocortisone acetate, only a very polar metabolite could be isolated. Behavior of this metabolite in counter-current distribution and paper chromatography resembled that of fraction IV-A. West *et al.* (2) injected 600 mg. of cortisol into a castrated, adrenalectomized and hypophysectomized patient and could not detect any estrogen in the urine. Sandberg *et al.* (14) administered a total of 200 mg. of cortisol over a three-day period to each of 3 castrated women and found no increase in urinary estrogen excretion. This observation led these authors to conclude that enhanced estrogen excretion occurring in women receiving ACTH was due to direct stimulation of adrenal estrogen secretion or to a peripheral conversion of nonestrogenic steroids of adrenal origin. It is evident that the questions concerning the origin of adrenal estrogens remain to be answered. Conversions of many other corticoids must be investigated. On the basis of our study, it may be stated that certain adrenal steroids can be converted to estrogens. This, however, does not exclude the possibility that the adrenal gland, under the stimulation of ACTH, will secrete estrogens.

The role of the conversion of cortisone to 11-oxygenated estrogens in

normal human subjects has not been ascertained. The significance of the conversion of cortisone to 11-oxygenated estrogens in an adrenalectomized patient with estrogen-dependent breast cancer is not known, since the effects of 11-oxygenated estrogens on the breast cancer cannot be assessed at this time.

Acknowledgment

This investigation was supported by Research Grant A-2878 from the National Institute of Arthritis and Metabolic Diseases. The authors wish to express their thanks to Dr. D. H. Peterson and Dr. J. A. Hogg, Upjohn Research Laboratories, Kalamazoo, Michigan, for the generous supply of 11β-hydroxyestrone, and to the National Institutes of Health for the supply of 4-C^{14}-cortisone acetate. They are also deeply indebted to Miss Carol Hilary and Miss Eva Bokany for their conscientious technical assistance.

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STUDIES ON THE THYROID ACTIVATOR OF HYPERTHYROIDISM*

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ABSTRACT

The thyroid activator of hyperthyroidism was distinguished from thyrotropin by its prolonged action in an assay for thyrotropin. It was sought in the serum of 101 persons and was found in: (*a*) 8 of 9 persons with exophthalmos and hyperthyroidism; (*b*) 19 of 25 persons without exophthalmos but with hyperthyroidism; (*c*) 19 of 23 persons with hyperthyroidism in the past but with persisting exophthalmos; (*d*) 11 of 14 persons with exophthalmos who had never had hyperthyroidism; and (*e*) only 1 of 25 persons with no history of either exophthalmos or hyperthyroidism. Included in these observations were 4 positive results on the serum of patients who had features of hypopituitarism due to pituitary-ablative surgery. Qualitative differences between thyrotropin and the thyroid activator of hyperthyroidism were found in: 1) serum fractionation studies by means of starch-block electrophoresis, chromatography on a column of diethylaminoethyl cellulose, and determinations by the percolation method of Bates; and 2) "half-life" experiments in mice, wherein the thyroid activator remained in the blood for several hours after injection, in contradistinction to the very short "half-life" of thyrotropin. It is suggested that the thyroid activator is of pathogenetic significance in Graves' disease and that it may be an entity distinct from pituitary thyrotropin.

EXCESSIVE secretion of thyrotropin has long been suggested as the cause of hyperthyroidism but assay of this hormone in the blood of persons with the disease has given conflicting results (1–3). Adams (4, 5) first provided evidence by bioassay that there may be in hyperthyroidism a circulating substance which stimulates the thyroid, but not in a manner identical with that of pituitary thyrotropin. His test animal, the guinea pig, responded maximally to standard thyrotropin in three to five hours but took about sixteen hours to respond maximally to serum from certain patients with hyperthyroidism. This phenomenon was confirmed (6, 7) in a similar assay using the mouse as a test animal. As described in the present paper, this thyroid activator was sought in the blood of a group of 101 persons and an attempt was made to correlate its presence with their clinical status, especially with regard to hyperthyroidism and exophthal-

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mos. In addition, further qualitative differences were observed between "normal" thyrotropin and the factor which, to facilitate discussion, has been termed "the thyroid activator of hyperthyroidism" (8).

METHODS

Details of the assay for thyrotropin and for the thyroid activator of hyperthyroidism have been published (6, 9). In brief, mice previously injected with I^{131} were injected intravenously with serum or other test substance and were thrice bled by venipuncture, once immediately before the intravenous injection, once two hours later, and a third time seven to nine hours later. The I^{131} in these (0.1 ml.) specimens of blood was measured and an increase, expressed as a percentage of the initial radioactivity, was used as the index of activation of the animal's thyroid. Thyrotropin in human or bovine pituitary extracts or serum from myxedematous patients caused a maximal increase after two hours whereas the injection of serum from many patients with hyperthyroidism caused a maximal increase only after about eight hours. When whole serum was tested the volume of the injection was 0.5 ml., but with extracts of serum it was usually 0.2 ml.; 4 to 6 mice were given each test injection. With injections of physiologic saline, inactive extracts of human blood, 5 per cent solution of human albumin, or serum from persons with spontaneous myxedema given thyroid, the eight-hour specimens of blood always exhibited a mean radioactivity not significantly greater than 100 per cent of the initial value. Consequently control studies were not carried out with every assay for the thyroid activator, but a delayed response was accepted as such only if it was significantly different (Student's "*t*" test, $P < 0.05$) from a response of 90 ± 9 (standard deviation). These figures were obtained by analyzing 20 consecutive assays of inactive solutions such as previously described.

Serum to be assayed was separated as soon as the blood clotted and was then stored at 4°C. until tested, except in the case of sera from 34 patients of Dr. E. P. McCullagh of the Cleveland Clinic, which were sent by air express and refrigerated on arrival; most sera were assayed within ten days of receipt. The decision about whether or not a patient had hyperthyroidism was made from the usual clinical criteria together with, in almost every case, ancillary laboratory data including estimation of the 24-hour thyroidal uptake of I^{131} and the concentration of protein-bound iodine in the serum. At the time blood was taken, 4 patients were hypothyroid as a result of treatment of hyperthyroidism (see subsequent data). Exophthalmos was diagnosed only when there was undoubted unilateral or bilateral protrusion of the eyes; any abnormality of eyelid retraction was discounted. No attempt was made to correlate assay results with estimates of the activity of the exophthalmos (that is, whether progressing or not).

Fractionation of 3-ml. specimens of serum was carried out by starch block electrophoresis (10), and of 10-ml. specimens by chromatography using a column of an anion-exchanger, *viz*, diethylaminoethyl (DEAE) cellulose (11), or by the "centrifugal method" of Bates and Condliffe (12) which is dependent upon the solubility of thyrotropin in aqueous solutions of alcohol and sodium chloride.

When "half-life" studies were carried out, USP Thyrotropin Reference Standard (2 milliunits in 0.5 milliliter of 0.9 per cent sodium chloride solution) or human serum (0.5 ml.) was injected intravenously into mice in groups of 6. For three days before this the animals were fed thyroid USP in their diet (final concentration, 0.066 per cent), so that endogenous thyrotropin would be thoroughly suppressed (9). At various time intervals after the injection of the thyrotropin or serum the groups were exsanguinated by percutaneous cardiac puncture under chloroform anesthesia. In this way, from a group

of 6 mice, a pool of 4 to 6 ml. of blood (drawn in heparinized syringes) was obtained. The plasma was separated immediately.

RESULTS

Sera containing the thyroid activator

Sera from 101 subjects were assayed and the data are summarized in Table 1.

Thyroid activator was found in these sera as follows: (*a*) in 27 (79 per cent) of 34 patients with active hyperthyroidism; (*b*) in 21 (75 per cent) of 28 patients with a history of hyperthyroidism who were euthyroid, all but 5 having exophthalmos; (*c*) in 11 (79 per cent) of 14 patients with the

TABLE 1. ANALYSIS OF ASSAYS OF SERUM FOR THE THYROID ACTIVATOR OF HYPERTHYROIDISM

Number	Clinical status		Positive results	
	Hyperthyroidism	Exophthalmos	Number	Per cent
9	Present	Yes	8	89
25	Present	No	19	76
23	Past	Yes	19	83
14	Never	Yes	11	79
5	Past	No	2	40
25*	Never	No	1	4
101 total			60 total	

* Myxedema, 10; post-op. thyroid cancer, 2; subacute thyroiditis, 1; acromegaly, 2; ?autonomous thyroid nodule, 1; normal 8.

exophthalmos characteristic of Graves' disease but no evident abnormality of thyroid function; and (*d*) only 1 subject among 25 with no history of hyperthyroidism and with no exophthalmos.

The values for these 60 positive assays are shown in Figure 1. The subjects were classified into the following groups: those with present or past hyperthyroidism with or without exophthalmos, those who never had hyperthyroidism but did have exophthalmos, and those who never had hyperthyroidism or exophthalmos.

In general, the patients whose serum elicited the greatest response in the assay had either severe exophthalmos or had hyperthyroidism prone to relapse after therapy. Thus 6 of the 13 values above 250 shown in Figure 1 were obtained on sera of patients with hyperthyroidism which had recurred more than once after treatment by subtotal thyroidectomy or by I^{131}, and 4 of these patients plus 4 others of this group had vision-threatening exophthalmos. However, this correlation was not absolute; for example, 1 patient whose serum elicited an assay response of 315 had but moderate exophthalmos and was euthyroid.

Four patients were hypothyroid as a result of treatment of hyperthyroidism—one permanently, after therapy with I^{131}, and the remainder temporarily, during treatment with propylthiouracil. In 3 of these 4 subjects, assays of serum indicated the presence of the thyroid activator (responses of 152, 243 and 325); in the fourth subject (hypothyroid from

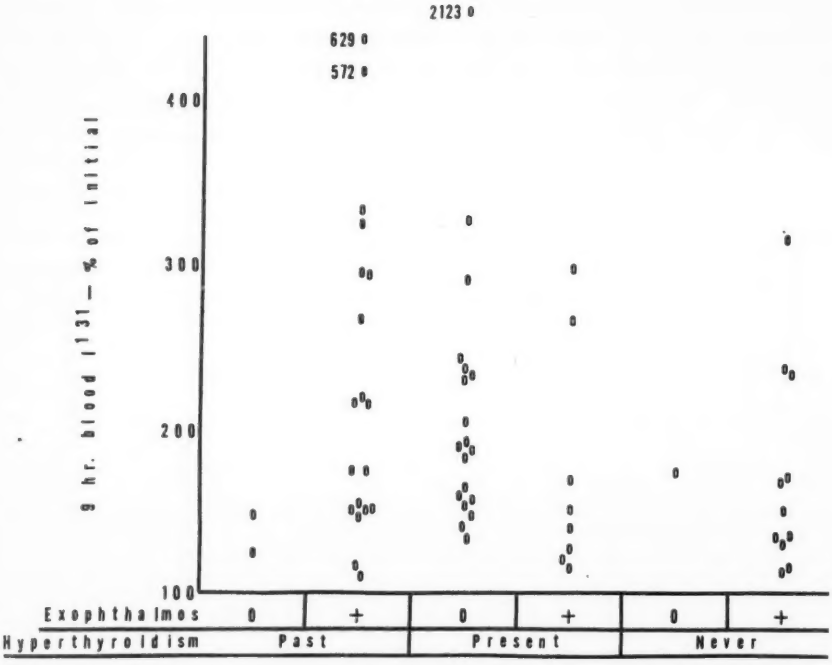

FIG. 1. Distribution of responses in 60 positive ($P < 0.05$) assays for the thyroid activator of hyperthyroidism. Each "0" represents the mean response in 4 to 6 mice injected with 0.5 ml. serum, and is measured as the percentage increase in circulating I^{131} seven to nine hours after the injection of the serum.

therapy with propylthiouracil) the response was typical of that caused by thyrotropin, *i.e.*, maximal at two hours after injection (two-hour response, 168; eight-hour response, 118). Among the 101 cases, the only other sera which elicited responses typical of thyrotropin came from patients with spontaneous myxedema.

Many of the positive results were obtained with serum from patients who were taking thyroid either as treatment for exophthalmos or during a "thyroid suppression test" (13). There was no apparent correlation between administration of thyroid and the degree of response in the assay of

serum; neither was there correlation between the degree of assay response and the concentration of protein-bound iodine in the patient's serum. However, the effect in a dosage greater than what is considered physiologic (200 mg. thyroid USP daily) was not investigated. In 2 cases, assays for thyroid activator were carried out before and after treatment with potassium iodide (plus propylthiouracil and reserpine); there was no significant change in the concentration of thyroid activator in either case.

Three patients (of Dr. E. P. McCullagh) with histories of hyperthyroidism had undergone pituitary stalk section for exophthalmos one to five years before serum was obtained for assay. A fourth patient (of Dr. J. C.

TABLE 2. ASSAY OF SERUM FROM PATIENTS WITH GRAVES' DISEASE AFTER SURGICAL ABLATION OF THE PITUITARY

Patient	Operation	9-Hour assay response*
#37	Pituitary stalk section	Repeat 244 ± 66 287 ± 158
#41	Pituitary stalk section	Repeat 144 ± 20 152 ± 50
#43	Pituitary stalk section	Repeat 116 ± 13 118 ± 10
#77	Hypophysectomy	Repeat 162 ± 23 181 ± 39

* Mean ± standard deviation. In no case was there a positive 2-hour assay response. The clinical state of these patients is described in the text.

Beck) had formerly had a partial thyroidectomy for Graves' disease and, more recently, hypophysectomy for metastatic mammary carcinoma. All required therapy for symptoms of hypopituitarism, and one—the subject of a separate report (14)—was suffering from a recurrence of hyperthyroidism. These 4 sera contained the thyroid activator (Table 2).

Three patients had goiter which clinically was obviously nodular and was known to have been present before the onset of hyperthyroidism. Two of these also had exophthalmos and their sera elicited responses at eight hours of 293 and 261; the serum of the third, who was without exophthalmos but had atrial fibrillation and cardiac decompensation, at eight hours elicited a response of 237.

In 2 female patients, one euthyroid and the other hyperthyroid, tests with radioiodine appeared to show that thyroid function was limited to a single circumscribed nodule, with no uptake of I^{131} by the remainder of the gland; administration of thyroid USP (200 mg. daily for three weeks) failed to suppress the I^{131} uptake, even in the euthyroid patient. Thyroid activator was not found in the serum of either of these women.

TABLE 3. HALF-LIFE OF INJECTED THYROTROPIN IN THE PLASMA OF MICE*

Assay of control plasma	Assay of plasma of mice injected with 2 mu. of thyrotropin			Assay of 0.5 mu. thyrotropin
	2 min.	15 min.	100 min.	
95 ± 9	218 ± 65	98 ± 14	84 ± 13	268 ± 53

* The mice were injected intravenously with 2 mu. of standard thyrotropin in 0.9 per cent sodium chloride and exsanguinated after the time intervals shown. Control animals were injected with 0.9 per cent sodium chloride solution. The response obtained with the injection of 0.5 mu. of standard thyrotropin is also shown. Results are the mean ± standard deviation of 6 responses in each case.

Half-life of thyrotropin and of the thyroid activator

When a dose of 2 milliunits (mu.) of USP Thyrotropin Reference Standard was injected intravenously into mice, the time after which thyrotropin could still be found in their blood was very short. The results are shown in Table 3; thyrotropin was easily assayed two minutes after injection but probably not after fifteen minutes, and certainly not after one hundred minutes. Since 2 mu. is a dose of thyrotropin beyond the linear portion of the dose-response relationship in this assay (9), the response to 0.5 mu. is also shown in Table 3 for comparison.

When 0.5 ml. of serum which contained the thyroid activator was injected intravenously into mice it could still be assayed in the blood of these animals eight hours later (Table 4). A similar response was observed with 3 other sera containing the thyroid activator, the half-life of which was estimated in this way.

Extraction of the thyroid activator from serum

When serum containing the thyroid activator was ultrafiltered through a filter with a pore size of less than 5 millimicrons, the thyroid activator

TABLE 4. HALF-LIFE OF THE THYROID ACTIVATOR OF HYPERTHYROIDISM IN THE PLASMA OF MICE*

Assay of control plasma	Assay of plasma of mice injected with 0.5 ml. of t.a. serum				Assay of original serum	
	2 min.	30 min.	4 hrs.	8 hrs.	whole	diluted 1:1
75 ± 25	270 ± 95	165 ± 20	144 ± 88	118 ± 11.8	323 ± 52	244 ± 47

* The mice were injected intravenously with 0.5 ml. of the human serum containing thyroid activator (t.a.), and exsanguinated after the time intervals shown. Control animals were injected with 0.9 per cent sodium chloride solution. The responses obtained with 0.5 ml. of the original t.a. serum, whole and diluted 1:1 with 0.9 per cent sodium chloride solution, are also shown. The results are the mean ± standard deviation of 6 responses in each case.

remained with the proteins and thus could easily be concentrated by this method.

Serum was fractionated by means of starch-block electrophoresis and four major fractions were obtained; these corresponded approximately to the conventional electrophoretic fractions—albumin, and the alpha, beta and gamma globulins. This fractionation procedure was carried out with 3 sera containing the thyroid activator. Activity was found in several fractions in each case (Table 5); proportionately, most activity was with the gamma globulins. The results were similar when 2 other sera from patients with hyperthyroidism were fractionated by means of chromatography using a column of diethylaminoethyl cellulose; again, the components roughly corresponded to albumin, and the alpha, beta and gamma globulins. When the chromatographic procedure was carried out with sera from 2 patients with spontaneous myxedema, all normal thyrotropic activity was found with the gamma globulins; this was similar to our previously reported results with the fractionation of serum from myxedematous patients by starch-block electrophoresis (10).

TABLE 5. ASSAY OF WHOLE SERUM AND FRACTIONS OF THAT SERUM FOR THE THYROID ACTIVATOR OF HYPERTHYROIDISM*

Fraction	Assay (mean ± standard deviation)
Whole serum	259 ± 46
Albumin	142 ± 24
Alpha globulin	139 ± 27
Beta globulin	102 ± 18
Gamma globulin	194 ± 42

* Serum was fractionated by starch-block electrophoresis. The fractions of serum named denote the major component of each fraction.

Attempts were made to fractionate 10-ml. specimens of serum from 3 patients with hyperthyroidism by the centrifugal modification of the percolation method described by Bates and co-workers (12), but little of the original activity was recovered and this was spread throughout all four fractions obtained and assayed. On three occasions, specimens of normal serum (15–25 ml.) were similarly treated and thyrotropic activity was found in the same fractions as described by the originators of the procedure (12), *viz*, 67 per cent ethanol, 5 per cent sodium chloride, 57 per cent ethanol, and 7 per cent sodium chloride.

A 10-ml. sample of a potent serum (eight-hour response 290) was subjected to extraction with a 2:1 chloroform-methanol solution (15), and the dried extract emulsified and assayed so that the equivalent of 2 ml. of serum was given to each of 5 mice. None of the original activity was found by this means. Perhaps of similar significance was the fact that there was no

apparent loss of activity in a few sera containing the thyroid activator when they were re-assayed after having been stored in a frozen state or having been lyophilized; both these procedures tend to denature lipoproteins (16).

Assays of Cohn fractions of normal human plasma

It was previously shown (9) that in Cohn fractions of (presumably normal) human plasma, obtained from the American Red Cross, thyrotropic activity was present in only fraction II. Solutions of approximately twice normal (17) concentrations of six Cohn fractions (I, II, III, IV-1, IV-4 and V) were made in 0.9 per cent sodium chloride solution, and assayed for the thyroid activator. No positive results were observed within eight hours in any of these assays.

Effect of thyroxine and of epinephrine

Solutions of sodium L-thyroxine (10 μg. in 0.2 ml. of 0.9 per cent sodium chloride solution) and of epinephrine (0.05 μg. and 1.0 μg., each in 0.2 ml of 0.9 per cent sodium chloride solution) were assayed in the same way as the sera. No response was observed with any of these injections either at two hours or at eight hours.

DISCUSSION

In the present series the thyroid activator was found in 59 of the sera from 76 patients with hyperthyroidism or exophthalmos or both, and only once in serum from 25 persons with neither condition. It is therefore tempting to assign to it a pathogenetic role in the hyperthyroid-exophthalmic complex commonly known as Graves' disease. Although these sera were assayed only for their ability to cause discharge of I^{131} from the mouse thyroid gland, 4 of them had been used in a series of experiments (7) showing that the discharge of I^{131} was not artefact but truly reflected thyroid activation. This was judged by an increased thyroidal uptake of I^{131}, an increase in the serum concentration of protein-bound stable iodine (I^{127}), and by histologic signs of augmented thyroid function. Although it remains an inference, it seems reasonable that such thyroid activation occurs in the human thyroid as a result of this factor in the blood.

There was no direct correlation between the thyroid activator in the blood of patients and the presence of exophthalmos, but the activator was found in 11 of 14 patients with exophthalmos and no evident thyroid dysfunction. Adams (5) suggested that this substance was possibly the "exophthalmos-producing substance" of Dobyns and Wilson (18); it would seem from the data reported here that coincident assays of sera for both the thyroid activator and exophthalmos-producing substance would be required to resolve this possibility.

The thyroid activator has been found twice in serum from normal persons (present report and ref. 19). Since it has also been found in persons with hyperthyroidism without exophthalmos and in some with exophthalmos without hyperthyroidism, this is not too remarkable. Only long-term studies will show whether or not its presence in such normal people signifies a potential for the onset of Graves' disease. It was not found in Cohn fractions of pooled human plasma. This may mean that it was not in the original plasma or was in too small concentration in the fractions to be assayed; on the other hand, the activity may well be lost in the processing of the plasma, in the same way as activity was lost by the treatment of potent sera with alcohol as described previously.

The thyroid activator was found in the sera of 3 of 4 patients who were hypothyroid after treatment of their hyperthyroidism. In the serum of the fourth patient "normal" thyrotropin was assayed in a concentration similar to that found in the serum of myxedematous patients, which would suggest a normal thyroid-pituitary interaction in this patient at that time. It has been shown (20) that a normal thyroid-pituitary relationship (as manifested by suppression of the thyroidal uptake of I^{131}) develops at a variable time after the treatment of hyperthyroidism with antithyroid drugs, and it has been suggested (21) that the development of such a normal relationship indicates the likelihood of a lasting remission of the hyperthyroidism. It may be that the presence of the thyroid activator instead of normal thyrotropin in the blood of a person who is hypothyroid from treatment of hyperthyroidism signifies the probability of relapse, should treatment be withdrawn at that point. Unfortunately the outcome of treatment in our 3 temporarily hypothyroid patients is unknown at present; 2 of these patients are still taking propylthiouracil and the third stopped only recently. It is interesting, however, that the patient permanently hypothyroid (whose serum elicited an eight-hour assay response of 243) had undergone a subtotal thyroidectomy for hyperthyroidism in 1947, and a recurrence had been treated with I^{131} in 1958.

Discussion frequently arises over the question of whether or not there are two (or more) types of hyperthyroidism—Graves' disease with diffuse toxic goiter and toxic nodular goiter. The present study, perhaps fortunately, contributes little to that controversy. As noted under "Results," 3 patients had known of their goiter before the onset of hyperthyroidism, but 2 of them had the exophthalmos of Graves' disease. The third of this group clinically might have been considered by some to have toxic nodular goiter; she was elderly, had a long-standing large nodular goiter, atrial fibrillation with cardiac decompensation and no exophthalmos. Her serum contained the thyroid activator. Perhaps of greater interest, however, were 2 other patients. One, a 23-year-old woman, was euthyroid but had a nodule of the thyroid, which by studies with I^{131} appeared to cause sup-

pression of function of the remainder of the gland (although no proof was obtained that the remainder of the gland was capable of function). This patient did not have the thyroid activator in her serum. The second patient was clinically hyperthyroid, but the thyroid "scan" was similar and her serum also did not contain the thyroid activator. We hope to study more patients of this type. If, as is proposed here, the thyroid activator is linked with the etiology of Graves' disease, it seems probable that these 2 patients and others, such as described by Wilansky and his colleagues (22), represent a group in whom the hyperactive portion of the thyroid gland is truly autonomous.

A major unsolved problem related to the thyroid activator is whether or not it is a form of pituitary thyrotropin or a distinct substance. Qualitative differences have been shown in the time course of the response in the assay animal, both with regard to the discharge (5, 6) and the uptake of I^{131} (7). Further, as described here for the studies in mice and briefly reported previously (8), the half-life of standard thyrotropin is much shorter than that of the thyroid activator, which is in agreement with results recently published by Adams for similar studies using rats (23). Although this difference might be related to the thyrotropin being injected in saline and the thyroid activator as whole serum, both Adams (5) and Munro (19) showed that pituitary thyrotropin retains its original activity in assays when dissolved in serum from a hyperthyroid person; this finding would tend to refute such an explanation for the difference in "half-life." Further differences were shown between the thyroid activator and thyrotropin in serum from patients with myxedema when both were extracted from serum by means of electrophoresis or chromatography; these results were similar to those reported by Munro (19). Attempts to use the procedure found to be successful by Bates and his associates (12) for the extraction of thyrotropin from normal human plasma, failed when applied to the extraction of thyroid activator from serum. The method was found appropriate, however, for the extraction of thyrotropic activity from normal serum. Similar apparent instability, or at least insolubility, of the thyroid activator in alcohol was described by Purves and Adams (24).

It must be admitted that none of these instances of difference between the thyroid activator of hyperthyroidism and thyrotropin conclusively proves that the substances are entirely distinct. The activator might differ only in the manner in which it is bound to the proteins of the plasma of the hyperthyroid subject. Data obtained with antisera to thyrotropin (25) tend to gainsay this concept; antisera to bovine thyrotropin were found to inhibit bovine pituitary thyrotropin and also thyrotropin in human serum (from patients with myxedema) but to have no effect on the thyroid activator in the serum of patients with hyperthyroidism. Further, standard

thyrotropin retained its original activity when dissolved in plasma containing the thyroid activator (5, 19). This would also indicate that the difference was not merely a matter of protein binding. Alternatively, the thyroid activator might be a molecule only slightly altered from normal thyrotropin; there is no evidence at present for or against this theory.

If it were accepted that the thyroid activator was not an abnormal thyrotropin but a separate entity, the problem of its site of origin would immediately arise. That it was found in the serum of 4 patients who had undergone surgery intended to ablate the pituitary and who had features of hypopituitarism intriguingly suggests an extra-pituitary source. However, pituitary stalk section is known to allow survival of hypophyseal cells (26), and one cannot be sure that the hypophysectomy in the fourth patient was complete. Moreover, specimens of serum have recently been obtained (through the kindness of Dr. David Becker) from a patient who had undergone hypophysectomy for metastatic carcinoma and who had a history of hyperthyroidism. Before operation, thyroid activator was found at nine hours in the assay of this patient's serum, but none was found after operation. Although this result does not necessarily imply that the thyroid activator originated in the pituitary, it certainly indicates the need for caution in any other interpretation. One definitive step which could be taken in the understanding of this problem would be the assay of pituitary tissue from a patient with hyperthyroidism.

Acknowledgments

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Addendum

Pituitary tissue has now been assayed. It was obtained at necropsy from a patient with Graves' disease whose serum in life contained the thyroid activator. When the pituitary was subjected to extraction with acetone or homogenization in water or 0.9 per cent sodium chloride solution, the resulting material contained only thyrotropin and no thyroid activator by assay in mice. Munro and his colleagues have made similar observations (27).

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ASSAY OF THE EXOPHTHALMOS-PRODUCING SUBSTANCE IN THE SERUM OF PATIENTS WITH PROGRESSIVE EXOPHTHALMOS*†

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ABSTRACT

Assays of the exophthalmos-producing substance (EPS) in the serum of patients with progressive exophthalmos were performed using *Fundulus hetroclitus*, an Atlantic minnow. The assay consists of measurement of the exophthalmic response in fish after injection of the patient's serum. A variety of factors affecting the responsiveness of the fish were partly controlled by the use of standard pituitary extract. The serum of certain patients produced pronounced exophthalmos in the fish. There was no strict correlation between the degree of response in the fish and the severity of clinical progressive exophthalmos. A better correlation was observed in patients early in the development of the disease. Several impressive responses were elicited by the serum of patients with Graves' disease preceding the onset of severe exophthalmos. Late in the course of progressive exophthalmos no high assay values were found. Mild degrees of response were occasionally elicited by the serum of normal subjects. Pituitary stalk section and cauterization of the anterior pituitary in a patient with extreme exophthalmos was followed by dramatic improvement in the eye symptoms and a fall in the EPS activity of the serum.

IN 1945 Albert (1) demonstrated that in the common Atlantic minnow (*Fundulus heteroclitus*, Linn.), exophthalmos developed following the administration of anterior pituitary extract. He used this response in Fundulus to test for the presence of exophthalmos-producing activity in a variety of pituitary preparations. In 1953 data were published (2) from our laboratory on the use of this fish to demonstrate the separation of the exophthalmos-producing substance (EPS) from the thyroid-stimulating hormone (TSH) of the pituitary. With refinements in the method it became possible to demonstrate EPS in amounts of pituitary extract as small as 15 micrograms. It therefore seemed appropriate to attempt to demonstrate EPS in the serum of patients suffering from severe active progressive

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exophthalmos. In 1954 there was published (3) from this laboratory a preliminary report on the finding of an exophthalmos-producing substance in the serum of some patients with severe progressive exophthalmos. Subsequently, others (4–6) demonstrated a similar response to such serum.

During the past nine years we have attempted to measure an exophthalmos-producing substance in 109 patients with progressive exophthalmos, in 22 normal control subjects, and in 3 samples[1] of pooled serum from normal subjects; 245 assays were performed. The purpose of this publication is to describe the results of these studies.

METHODS

Fundulus in groups of 8 to 12 were placed in stainless steel tanks containing fresh water that had been filtered through carbon. The tanks were kept in a temperature-controlled room at 14° C. in the presence of continuous light. A colored thread was tied through the lower lip of each fish so that identification of individuals was possible. Owing to the lateral position of the eyes, the distance between the two corneal surfaces could be measured; any increase in the distance, representing a protrusion of the globes, was recorded (Fig. 1). The intercorneal distance (ICD) in each fish was measured to 0.1 mm. by sighting in a parallel fashion along special calipers arranged just above the eyes of the fish, as shown in Figure 1. Readings were taken from a vernier scale which could not be seen until after the setting had been completed. Measurements on each fish were made before, and at intervals of six to twenty-four hours following administration of the serum to be tested. The exophthalmos which developed in each fish was expressed as the percentage increase in ICD. Each series of measurements was made by the same observer. Since the initial intercorneal distance of these fish ranged from 10 to 12 mm. and the accuracy of the measurements was within 0.3 mm., changes in the ICD of greater than 3 to 4 per cent were detected with reasonable reliability.

The material to be tested was injected into the coelomic cavity of the fish by the introduction of a #25 needle into the vent and through the wall of the bowel. The volume of injection material which could thus be accommodated at one time was little more than 0.25 ml. in the smallest fish used. Because this volume of serum usually produced small responses compared with potent pituitary extracts, whole human serum was given in 3 injections over a 24-hour period, making a total of 0.75 ml. per fish.

Several types of control observations were made. Because of seasonal and other variations in the responsiveness of the fish, a dose of 256 μg. of a standard pituitary extract in 0.25 ml. of physiologic sodium chloride solution was injected into each of a group of 10 fish as a standard of reference for the interpretation of the responsiveness of the fish each time an assay of serum was attempted.

A second type of control consisted of observations on pooled normal sera. In 3 separate experiments the sera of 10 normal subjects were pooled so that any special constituent in any one serum was effectively diluted by the other 9 sera. The responses elicited by the 3 different pools of normal sera were tested in separate series of fish.[2]

The third type of control represented the response to individual sera from normal persons. At a time when the fish were demonstrated to be responding moderately to exceptionally well, the sera of 22 normal subjects were tested.

[1] Previously reported by Dobyns and Wilson (3).

[2] Previously reported by Dobyns and Wilson (3).

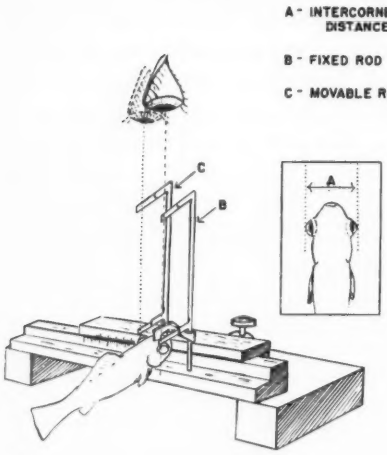

FIG. 1. Method of measuring intercorneal distance in fish.

The fish is placed so that the highest point of the corneal surface of one of its eyes is in line with the two sighting posts on rod B. The two posts on movable rod C are then aligned with the highest point on the corneal surface of the other eye. Intercorneal distance A is read from a vernier scale. Multiple observations detect changes in the prominence of the eyes.

FACTORS OF IMPORTANCE IN THE ASSAY

Dose-response curve

Before describing the application of the assay method to clinical problems, it is necessary to discuss factors which may be important in this assay. The same pituitary extract, relatively rich in EPS, has been used as the standard for all observations during the past nine years. Several attempts have been made to define a dose-response curve with this material. Fish were divided into groups of 10; injections consisted of the standard pituitary extract at one of eight dose levels, or physiologic sodium chloride solution. Three such experiments were performed at the same time of the year, when the fish were responding well. The results (Fig. 2) demonstrate: 1) the variability of individual responses, and 2) a rough correlation between increasing response and increasing dose.

When saline solutions of pituitary extracts are injected into fish, the time required for the exophthalmic response to occur appears to depend upon the degree of purification of the extract. Presumably the more rapid response is attributable to the removal of some of the excess protein from the extract. The exophthalmic response to the pituitary extract used in these experiments develops within four to twenty-four hours. It is sustained for varying lengths of time, dependent upon the amount of extract given. On the other hand, the response to injected serum develops very slowly and is sustained over a considerable period of time (Fig. 3). Presumably, absorption of the protein of the serum and its active ingredient is slow. Thus, the response curves reach less significant levels. Since the fish exhibit their maximal level of response at different times following the in-

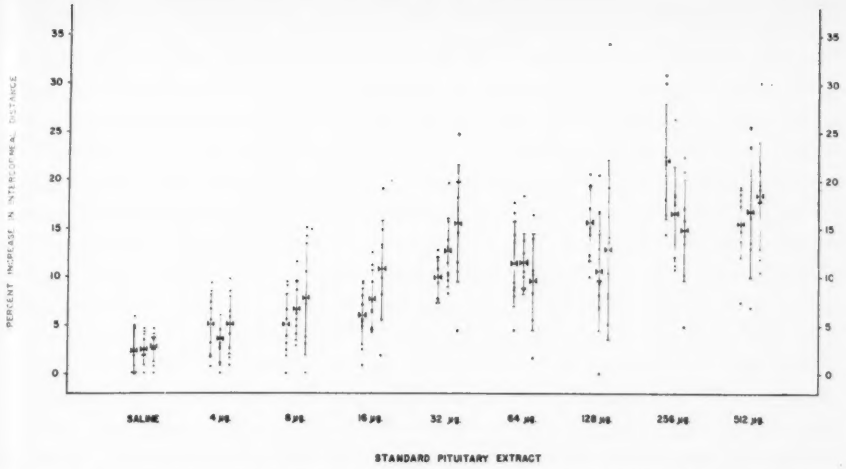

FIG. 2. Three attempts to establish a graded dose-response relationship, using serial dilutions of a standard pituitary extract.

Each dot in a vertical row represents the maximal response of an individual fish in a group of 10. The average of the maximal responses and the standard deviation are also shown. The data are derived from 3 separate experiments. The first, second and third groups of fish at each dose level represent respectively the 3 series. Wide variations in individual responsiveness are observed.

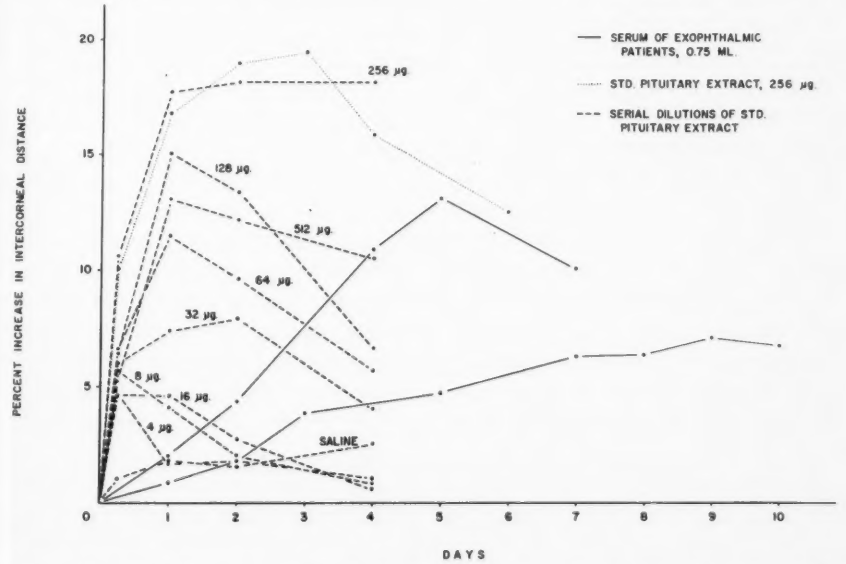

FIG. 3. Comparison of the exophthalmic responses produced by varying doses of a standard pituitary extract and by sera rich in EPS.

Each dot represents the average of the maximal responses in a group of 10 fish observed over a certain period of time (days). See text for discussion.

jections, the maximal increase in ICD attained in each fish, regardless of time, is the response of record. The response to any serum is the average of these maxima for all fish in the group, and is expressed as a percentage increase in ICD.

Even though the exophthalmic response in some fish in each group may be impressive, the response in others may be nil. In some groups of fish the exophthalmos may occur in only one or two fish, but this response is so striking that it is readily identifiable without the application of a precise method of measurement. A failure of measurable response among a small group of test fish thus gives no assurance that EPS is absent, but the development of striking exophthalmos in an occasional fish leads to the supposition that there may have been some exophthalmos-producing activity in the material injected. The causes of failure of response have been extensively explored and will be the basis of another report. Gradually, as experience has been gained, a variety of technical and biologic factors related to the response have come to light. These have been given increasing attention but require much more exploration before an entirely satisfactory method of assay is available.

Seasonal variation

Abundant data collected over nine years are now available to show that there is a seasonal variation in the responsiveness of the fish. There is a noticeable tendency toward poor responses in March, April and May, and toward much better responses during the winter months. However, some poor responses occur among the good ones in the winter months, but the responses are uniformly poor in the spring. The responses are not sufficiently uniform from year to year, even in the winter months, to predict the results with certainty and thus establish seasonal correction factors.

If fish respond differently with the seasons, the response of any group of fish given injections at one time of year cannot be compared with the response at another time of year unless some correction is made. Therefore, the average of the maximal responses caused by the injection of serum was related to the average of the maximal responses produced by the standard dose of pituitary extract given to a group of fish at the same time the serum was tested. The following formula was used:

$$\frac{\text{Average maximal response (serum)}}{\text{Average maximal response (standard pituitary extract)}},$$

expressed as a percentage.

Tests of reproducibility

To examine the method of assay and the correction for variability in the magnitude of the response, two tests of reproducibility were considered.

The first was the comparison of the results of assays made on the serum from two samples of blood obtained from the same subject at an interval of a few days or weeks. Data were considered only on subjects in whom no change in clinical status or therapeutic regimen had occurred. Such assays performed six weeks or less apart in 6 subjects who remained clinically unchanged are listed in Table 1. The agreement was considered good in 5 and fair in 1.

TABLE 1. ASSAYS OF EPS IN SERUM OF BLOOD SAMPLES PROCURED FROM EACH OF 6 SUBJECTS WITHIN A PERIOD OF SIX WEEKS, DURING WHICH THERE HAD BEEN NO SIGNIFICANT CLINICAL CHANGE

Name	Response* $\left(\frac{\text{serum}}{\text{standard}}\ \%\right)$	Dates
B.B.	35.9	Dec. 11, 1958
	39.5	Jan. 16, 1959
R.G.	38.4	July 29, 1955
	25.8	Sept. 20, 1955
R.S.	41.1	Sept. 16, 1958
	30.9	Nov. 3, 1958
N.Lo.	35.4	Oct. 26, 1954
	19.6	Oct. 27, 1954
B.M.	59.5	Nov. 11, 1955
	53.5	Nov. 29, 1955
R.P.	26.0	Jan. 12, 1956
	17.6	Feb. 22, 1956

* Response produced by serum, expressed as a percentage of the response produced by a standard pituitary extract.

A second way to appraise the reproducibility of the response, and incidentally to appraise stability during storage at −22° C., involves assays on aliquots of the *same* sample of serum after short lapses of time. Single samples of serum were divided into two aliquots and assayed at different times, the interval between the assays being two weeks to four years. Table 2 shows that sera with high exophthalmos-producing activity can be stored for as long as twenty months in the frozen state and still retain some of their potency.[3] Since a reasonably good exophthalmic response was demonstrable after several months of storage, it was thought that assays made on aliquots of samples stored six weeks or less might be used to appraise the reproducibility of the method. Re-assay within six weeks gave moderately comparable results (Table 2). Since these values often showed an increase

[3] Data on all sera stored for longer than five months were eliminated from the tabulations in all other experiments described here.

TABLE 2. RE-ASSAYS ON ALIQUOTS OF THE SAME SERUM, TO TEST REPRODUCIBILITY OF THE METHOD AND STABILITY OF EPS DURING STORAGE

Name	Response* $\left(\frac{\text{serum}}{\text{standard}}\ \%\right)$	Dates	Interval
O.B.	61.1 34.7	July 26/54 Aug. 5/54	2 wks.
M.G.	28.2 42.0	Sept. 6/57 Sept. 23/57	2 wks.
P.T.	107.0 75.2	June 18/54 July 26/54	4 wks.
M.R.	96.4 84.6	June 18/54 July 26/54	5 wks.
C.B.	40.0 37.6	Nov. 25/57 Jan. 2/58	6 wks.
C.M.	38.3 27.5	Oct. 12/55 Nov. 28/55	6 wks.
W.D.	14.3 12.2	Feb. 25/57 Apr. 18/57	2 mos.
M.S.	47.1 65.4	May 18/54 July 26/54	2 mos.
M.T.	42.8 56.4	May 12/54 Aug. 5/54	3 mos.
A.F.	20.5 21.0	Oct. 28/57 Feb. 10/58	4 mos.
E.H.	25.5 41.8	May 4/55 Sept. 8/55	4 mos.
C.O.	60.4 43.5	June 8/54 Oct. 24/55	4 mos.
M.S.	18.8 53.0	Oct. 29/54 Oct. 24/55	12 mos.
N.G.	30.6 22.8	Sept. 21/54 Nov. 30/55	14 mos.
L.H.	15.4 16.8	Dec. 27/56 Feb. 10/58	16 mos.
B.R.	32.4 41.1	Apr. 16/54 Nov. 7/55	19 mos.
H.B.	27.9 22.2	Mar. 26/54 Nov. 8/55	20 mos.
F.K.	35.7 4.3	Sept. 21/54 Mar. 4/57	2½ yrs.
K.M.	47.5 8.4	Aug. 4/54 Feb. 5/58	3½ yrs.

* See footnote to Table 1.

rather than a decrease after storage, the question of deterioration within a few months seemed less important than the variables in the method of assay. In the 2 sera that had been stored for two and a half and three and a half years respectively, the exophthalmos-producing activity had disappeared.

RESULTS—APPLICATION TO CLINICAL PROBLEMS

Relationship to stage of clinical exophthalmos

The serum of some patients with progressive exophthalmos caused a pronounced increase in the prominence of the eyes of the fish. The results of all assays in relation to the condition of the patients' eyes are listed in Table 3. The subjects were classified into 3 major groups as follows: 1. *Graves' disease without progressive exophthalmos*, the only eye signs being lid retraction, lid lag and stare. 2. *Progressive exophthalmos* with such eye signs as lacrimation, photophobia, injection of the conjunctival veins, pressure and pain behind the globes, and increasing proptosis; this group was subdivided into mild, moderate, and severe with respect to severity of the eye symptoms, without knowledge of the assay data and irrespective of the state of thyroid function. 3. *Normal subjects.*

In general, the serum of patients with progressive exophthalmos produced more exophthalmos in the fish than did serum from patients with Graves' disease without exophthalmos or serum from normal controls. Considering the number of determinations in each group (Table 3), there were fewer low values and more very high values for the assays on serum

TABLE 3. ASSAY OF EPS IN RELATION TO THE CLINICAL STATE OF THE PATIENTS' EYES*

Response† $\left(\frac{\text{serum}}{\text{standard}}\ \%\right)$	Normal controls	Graves' disease			
		Without exophthalmos	With exophthalmos—severity		
			Grade I	Grade II	Grade III
00.0–14.9	7	6	3	2	0
15.0–29.9	10‡	8	17	9	16
30.0–44.9	7	4	10	12	16
45.0–59.9	3	1	6	3	8
60.0 and over	0	0	5	5	4
Total	27	19	41	31	44

* 162 determinations, not including samples stored for more than five months.
† See footnote to Table 1.
‡ In addition to these 10 individual sera, the 3 samples of pooled serum all fell in this category.

from patients with progressive exophthalmos than on serum from patients without exophthalmos.

It is possible that, although the exophthalmos in a given patient may be extreme, the severity may actually be diminishing and consequently the EPS level in serum might be low. It is also possible that in a patient with Graves' disease without exophthalmos, exophthalmos may develop suddenly and progress rapidly. Indeed, the sera of 3 patients with Graves' disease, assayed before progressive exophthalmos began, did show high EPS titers several weeks or months before the eye symptoms were recognized. The production of exophthalmos in the fish by the sera of some normal subjects was unexpected. In none of these normal subjects did Graves' disease or exophthalmos subsequently develop. In contrast to the moderate exophthalmic response occasionally produced in fish by individual samples of normal serum, the responses to pooled normal sera were consistently low (Table 3). In view of the occasional significant responses produced by some seemingly normal sera, the question of the specificity of the response must be further explored. On the other hand, it may be that all patients with progressive exophthalmos will not show EPS in their serum because of the time in the course of the disease when the observation is made (7).

The development of exophthalmos may be very insidious, and since secondary mechanical factors may contribute to the symptoms, it is difficult to appraise the clinical evidence for the presence of the causative factor of exophthalmos. For instance, a patient with exophthalmos manifesting the angry aspects of the disease may not show signs of clinical improvement as soon as the causative factor begins to abate. Hence a low EPS assay value might be found in a patient with what seems to be active disease. Conversely, there may be times when the causative factor is present in excess, yet the clinical manifestations of the disease are barely evident. The patients described here were in all stages of the disease from the pre-emergence state to the final arrested state, so an attempt was made to classify the clinical state of the exophthalmos into 3 categories, *i.e.*, regressing, stationary, and progressing regardless of severity. Decision was based on the course of the symptoms and the measurements immediately before the procurement of serum and an appraisal of changes in the clinical signs soon thereafter. Table 4 shows that no strict correlation could be demonstrated between the results of assays and the clinical appraisal of regression or progression of the disease.

Assuming that the causative factor might be more easily demonstrable during the early development of the disease than later, the data obtained on patients within the first six months of the onset of progressive exophthalmos were analyzed (Table 5). The incidence of EPS in serum was

TABLE 4. EPS ASSAYS IN RELATION TO THE CLINICAL STATUS OF THE EXOPHTHALMOS

Response* $\left(\frac{\text{serum}}{\text{standard}}\ \%\right)$	Exophthalmos		
	Regressing	Stationary	Progressing
00.0–14.9	2	7	1
15.0–29.9	14	18	15
30.0–44.9	8	17	10
45.0–59.9	2	4	6
60.0 and over	1	1	3
Total	27	47	35

* See footnote to Table 1.

slightly higher in this group than in the total group, and was considerably higher than in the controls. Assay values were lower among patients whose disease had been present for several years. These findings support the belief that clinical investigations to explore the cause of progressive exophthalmos should be confined to those patients who are in the early phases of the disease.

Relationship to thyroid function

From the clinical viewpoint, the effect of thyroid function on the course of progressive exophthalmos is not clear. Progressive exophthalmos may occur with severe hyperthyroidism or with a euthyroid state. Exophthalmos appears to be merely one of the facets of the Graves' syndrome and may precede, accompany or follow the hyperthyroidism. Presumably the water storage and gain of body weight associated with the development of hypothyroidism exaggerate the exophthalmos. Fortunately, most patients with progressive exophthalmos and hyperthyroidism experience relief from

TABLE 5. COMPARISON OF ASSAYS OF EPS EARLY AND LATE IN THE DEVELOPMENT OF PROGRESSIVE EXOPHTHALMOS*

Response† $\left(\frac{\text{serum}}{\text{standard}}\ \%\right)$	Number of patients with onset of exophthalmos—			
	within 6 mos.	2.0 to 3.9 yrs.	4.0 to 5.9 yrs.	6.0 or more yrs.
00.0–14.9	0 (00.0%)	3	2	1
15.0–29.9	10 (32.3%)	8	2	4
30.0–44.9	11 (35.5%)	6	1	3
45.0–59.9	7 (22.5%)	4	1	0
60.0 and over	3 (9.7%)	4	0	0
Total	31 (100.0%)	25	6	8

* Compare with Table 3.
† See footnote to Table 1.

the ocular condition when the hyperthyroidism is corrected. Unfortunately, however, the correction of hyperthyroidism does not immediately stop the cause of Graves' disease, so that recurrence of hyperthyroidism or further progression of exophthalmos may occur. The rise in the level of TSH after thyroidectomy in animals has led to the supposition that such a rise in man might make the eye problem worse, but it has been shown that pituitary EPS is a substance distinct from TSH and that TSH *per se* does not produce exophthalmos (2). It is for this reason that the exophthalmos-producing quality of the serum of patients with progressive exophthalmos before and after correction of hyperthyroidism has been considered here. Two methods of treatment of hyperthyroidism were used—thyroidectomy and radiation with I^{131}. Assays were made both before and after the correction of hyperthyroidism. As might be expected from clinical observations, no obvious trend toward either a rise or fall in assay values among this group of patients could be demonstrated. Until the method of assay is more firmly established, however, definite conclusions cannot be drawn.

Correlation of serum EPS values with course of clinical exophthalmos

Illustrations of the clinical course of severe progressive exophthalmos in relationship to the values for EPS found in a series of assays are shown for 3 patients in Figures 4, 5 and 6. The patient represented in Figure 4 had extremely severe exophthalmos, with impending need for orbital decompression in order to save sight. The symptoms began to abate shortly after recovery from hyperthyroidism (radioiodine therapy). Except for some impairment of function of the extraocular muscles, recovery was satisfactory. Although all of the assay responses were classified as minimal, the results of the multiple assays paralleled the changes in the clinical state of the disease.

Figure 5 illustrates the course of the disease in a patient who suffered from rapidly developing extreme exophthalmos which regressed when hydrocortisone was given. In this case the EPS assay values also reflected the clinical change in the eyes, although the assay response was not pronounced even at the height of the disease.

Another patient[4] (Fig. 6) required surgical intervention because of extreme exophthalmos. Within ten days following surgical division of the stalk and cauterization of the pituitary, the exophthalmos subsided dramatically. The exophthalmic responses to the serum immediately before and four days after stalk section were among the highest observed in any patient. The responses on subsequent assays were much reduced, coinci-

[4] Observations on this patient were made possible through the kindness of Dr. E. P. McCullagh, Cleveland Clinic, Stalk section was performed by Dr. W. James Gardner. Published as Case #8 by McCullagh, Clamen and Gardner (8).

dent with the dramatic clinical recovery. Assays were all carried out within a period of four weeks.

Pretibial myxedema

Localized pretibial myxedema occurs quite frequently in patients suffering from progressive exophthalmos. There is great similarity between the

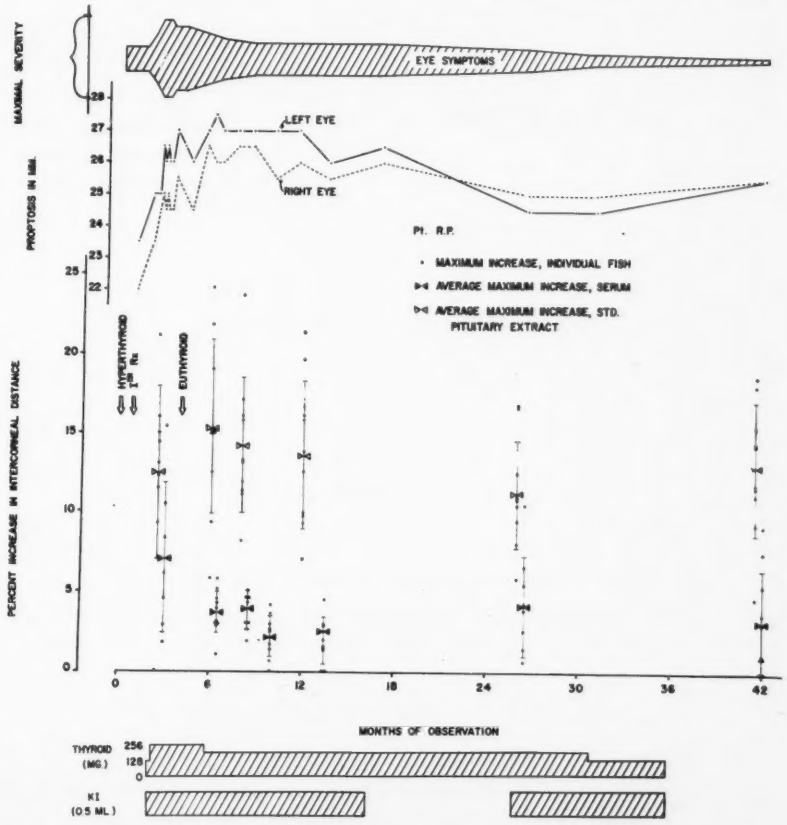

FIG. 4. A series of EPS assays made on the serum of a patient with severe progressive exophthalmos, demonstrating the relationship of changes in the intercorneal distance in fish to the patient's thyroid status, relevant medication and eye symptoms. The patient's eye symptoms were severe but the values for EPS in serum were low. The greatest assay value, although not remarkably high, was obtained at the time the eye symptoms were most severe.

The responses here and in the following figures are expressed both individually and as the average maximal increase in each group of fish receiving serum. The responses to the standard pituitary preparation are similarly shown. Thus the reader may observe the degrees of response rather than the ratios used in the tables.

nature of the pathologic changes in the retrobulbar tissue and that of the lesions on the lower legs. Therefore EPS assay data were analyzed with respect to the presence of pretibial myxedema. Neither the degree nor the incidence of exophthalmic response was any greater in patients with pretibial myxedema than in those without this lesion.

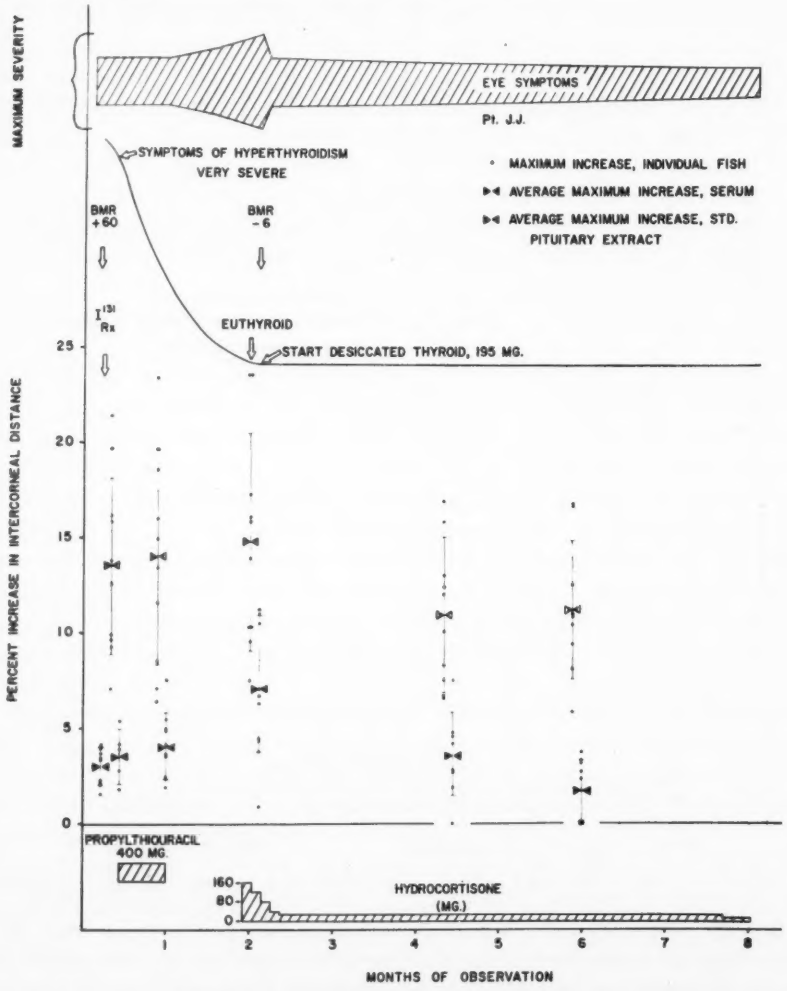

FIG. 5. A series of EPS assays on the serum of a patient with severe progressive exophthalmos, illustrating the relationship of changes in the intercorneal distance in fish to the patient's thyroid status, relevant medication and eye symptoms. Assay values rose as the patient's exophthalmos increased, coincident with the disappearance of hyperthyroidism.

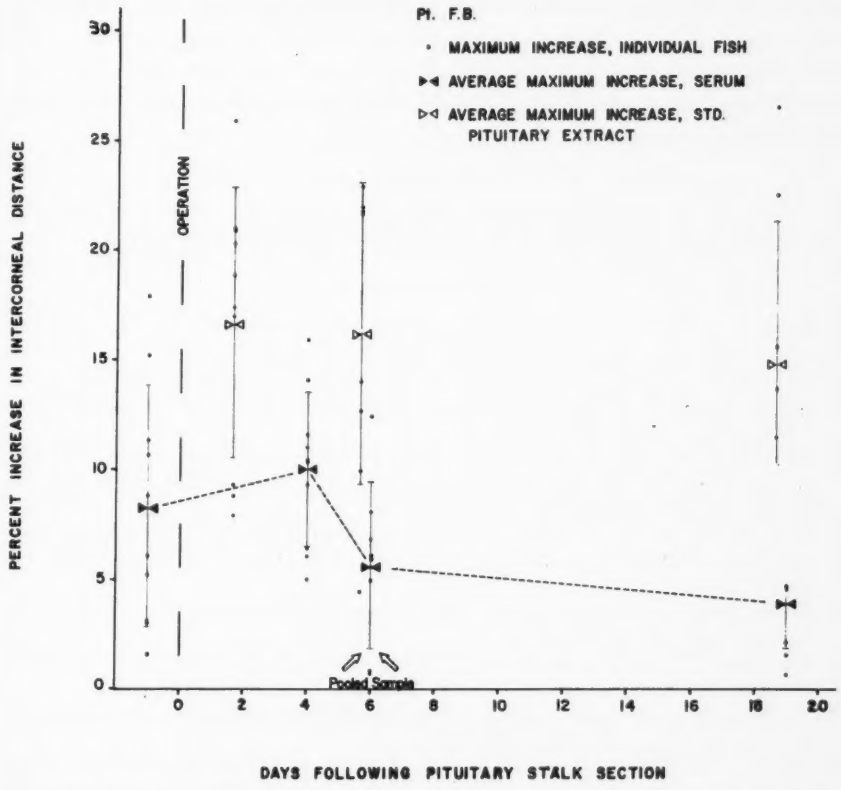

FIG. 6. Fall in the titer of serum EPS following section of the pituitary stalk. Coincident with the fall in assay values there was a dramatic improvement in the exophthalmos.

COMMENT

The data presented here have been collected over nine years and are not entirely uniform in character. The basic design of the method of assay has remained the same, but variations not fully appreciated at first have received progressively more attention as time has passed. The material for study has been relatively abundant but the circumstances of collecting and testing the serum have been variable. This does not, however, negate the observation that an exophthalmic response in the fish resulted from the administration of certain sera, the greatest response being elicited by the sera of patients with progressive exophthalmos. Any method which may be used on serum to concentrate the active substance of the serum and reduce the unrelated protein will be of great value. The lack of uniformity of the response in individual fish, as well as the seasonal variation, must be better

controlled. Perhaps a biologic study of the fish may lead to methods for enhancement of the response or even to basic considerations which will shed light on the nature of exophthalmos in man. Until some of these improvements are accomplished, the interpretation of EPS assays will remain uncertain.

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BIOSYNTHESIS OF STEROIDS BY TESTICULAR TUMORS COMPLICATING CONGENITAL ADRENOCORTICAL HYPERPLASIA*

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ABSTRACT

In vitro studies of bilateral testicular tumors occurring in a boy with congenital adrenocortical hyperplasia showed that these tumors synthesized large amounts of 17α-hydroxyprogesterone. The accumulation of 17α-hydroxyprogesterone in amounts greater than normal for either testicular or adrenal tissue is attributed to the low degree of activity of both 21-hydroxylase and side-chain splitting enzyme in the testicular tumors. The excretion of 17-ketosteroids was elevated in this patient. It is suggested that the excess urinary 17-ketosteroids were formed at some site other than the testicular tumors, from precursors which were part of the tumor secretion. This hypothesis is supported by the low side-chain splitting activity found in the tumors, the increased levels of 17α-hydroxyprogesterone in spermatic vein blood after administration of ACTH, and the fall in both 17-ketosteroid and pregnanetriol excretion after bilateral orchidectomy.

STUDIES of steroid biosynthesis by testicular tumors in a 16-year-old boy with congenital adrenal hyperplasia and early virilization yielded the information reported here. The clinical aspects of the case were described in a recent paper in this journal (1); the early history was previously discussed elsewhere (2, 3).

MATERIALS AND METHODS

Tissues. The right testis of the virilized boy was removed June 19, 1959, and weighed 55 Gm. It was kept deep-frozen until incubation studies were carried out. The left testis, removed September 29, 1959, weighed 100 Gm. and had obviously increased in size during the three months since right orchidectomy. Immediately after removal it was packed in dry ice and sent to this laboratory. Both specimens were thawed, and tissue incubation was begun October 3, 1959. The two specimens appeared similar histologically, each testicular mass consisting almost entirely of tumor tissue.

Spermatic vein blood. Spermatic vein blood was collected immediately prior to left

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orchidectomy. With a plastic catheter in the spermatic vein, blood was withdrawn at the following times: 1) Before administration of corticotropin (ACTH), 20 ml. was collected for use as a control sample. 2) After the intravenous administration of 6.5 units of ACTH, 7 ml. was collected. 3) After the intravenous injection of a total of 7.5 units of ACTH, 4 ml. was collected. Each of the 3 frozen plasma samples was sent to this laboratory to be analyzed.

The 3 samples of plasma were subjected to extraction with chloroform. After removal of the solvent the extracts were chromatographed in a defatting hexane-formamide system, and immediately thereafter the same chromatogram was developed with hexane-benzene in parallel with a strip containing testosterone, 11β-hydroxy-4-androstenedione, and 4-androstenedione. The ultraviolet (UV) absorbing materials were detected with a Haines UV scanner (4). Iodine-potassium iodide (I_2KI) and Zimmermann color spot tests were also made on narrow strips of the chromatogram. UV-positive areas were then cut out and the material eluted with 2 ml. of vacuum-distilled methanol. Determinations of UV spectra and quantitative estimations were carried out in a Beckman spectrophotometer, model DK_2, using the eluate obtained from the corresponding zone of a blank paper strip as blank. The amount of steroids was calculated as described by Cruz and Gomez-Mont (5). Sulfuric acid spectra were developed by Zaffaroni's method (6). Acetylation of sample 2C (see Fig. 2 and Results) was performed with a mixture of acetic anhydride/pyridine (1:5, v/v) at room temperature for twenty-four hours.

Incubation. The *in vitro* methods of study of steroid biosynthesis used were those previously described (7–9). In these previous publications a fairly good correlation was shown between the *in vitro* incubation products and *in vitro* hormone production.

The testicular tumors were homogenized separately in Krebs-bicarbonate-buffer/bovine serum, 1:1, previously adjusted to pH 7.4, and containing 40 mM of nicotinamide. An amount of homogenate equivalent to 200 mg. of tissue was placed in glass-stoppered Erlenmeyer flasks, to which radioactive substrates dissolved in 0.2 ml. of ethanol had previously been added. Adenosine triphosphate (0.4 mM), diphosphopyridine nucleotide (0.4 mM), triphosphopyridine nucleotide (0.1 mM), glucose-6-phosphate (0.5 mM), and fumaric acid (1.0 mM) were used as co-factors, and 1 mg. of glucose-6-phosphate dehydrogenase was added per flask. Incubation was carried out at 36.5° C. for three hours in an atmosphere of 95% O_2 – 5% CO_2.

Substrates. Progesterone-4-C^{14}, 17α-hydroxyprogesterone-4-C^{14}, 11-deoxycorticosterone-21-C^{14}, 4-androstenedione-4-C^{14}, and testosterone-4-C^{14} were used at levels of 25 and 100 mμmoles in different flasks.

Extraction. At the end of the incubation period, 1.0 ml. of 1 *N* hydrochloric acid was added to the medium and the steroids were extracted with chloroform/ether, 1:4. The pooled extracts from each flask were concentrated under nitrogen and aliquots plated to determine recovery. Recovery ranged between 87 and 93 per cent.

Chromatographic analysis. The extracts were chromatographed on paper by the use of Zaffaroni systems (10). Radioactive steroids were located on the paper strips (11) and the quantitative determinations were made by the method reported previously (12). By this procedure the sensitivity of detection was 0.01 per cent conversion from 100 mμmoles of substrate, and 0.05 per cent conversion from 25 mμmoles of substrate.

The steroids isolated directly by paper chromatography or by quantitative acetylation and subsequent paper chromatography were finally identified by the formation of various derivatives and comparison of running rates in various solvent systems with known standards added to the crude extracts, or at different stages during the isola-

tion and purification of the radioactive metabolites. When possible, the purified compounds were recrystallized to constant specific activity in the presence of 10 mg. of the corresponding authentic steroid in order to establish identity.

RESULTS

The two testicular masses formed virtually the same compounds during incubation; but the steroid biosynthetic activity of the left mass, the larger and more recently removed of the two, was greater than that of the right (Table 1).

TABLE 1. RELATIVE STEROIDAL ACTIVITY OF THE TWO BILATERAL TESTICULAR TUMORS INCUBATED WITH 100 MμMOLES OF PROGESTERONE-4-C^{14} IN THE PRESENCE OF 200 MG. OF HOMOGENIZED TISSUE

Chromatographic zone*	Amount of steroid (mμmoles)	
	Right testicular tumor	Left testicular tumor
A	11.2	14.0
B	0.8	3.5
C	29.5	37.2
D	—	1.7
E	58.5	43.6

* A = More polar steroids (including 4-pregnene-17α,20α-diol-3-one).
B = 6β-Hydroxyprogesterone and 4-pregnene-17α,20β-diol-3-one.
C = 17α-Hydroxyprogesterone, testosterone, and 11-deoxycorticosterone.
D = 4-Androstenedione and 4-pregnene-20α-ol-3-one.
E = Progesterone.

When progesterone-4-C^{14} was incubated with an homogenate of 200 mg. of the left tumor, the compounds listed in Table 2 were isolated. Besides material remaining at the origin, there were only two compounds the structure of which could not be determined with fair probability. The criteria for identification of the others are given in Table 3.

Incubation in the presence of progesterone-4-C^{14} yielded a high degree of 17α-hydroxylase activity; as much as 100 mμmoles of progesterone was not enough to maintain enzyme saturation throughout the incubation period when a 200-mg. sample of tissue homogenate was used. Substrate concentration studies with this system[1] have shown that maximum velocity is maintained if the concentration of progesterone remains above 5 μM (50 mμmoles per 10 ml.). The second enzyme in importance was 20α-hydroxydehydrogenase, which reduced the 20-keto group. Both 4-pregnene-20α-ol-3-one and 4-pregnene-17α,20α-diol-3-one were formed. Two other hydroxylases competed with the 17α-hydroxylase and 20α-hydroxydehydrogenase, namely, 21-hydroxylase and 6β-hydroxylase. These accounted for the formation of small amounts of 11-deoxycorticosterone and 6β-hydroxyprogesterone.

[1] Manuscript in preparation.

The extremely small amounts of 4-androstenedione and testosterone formed during the incubation period indicated a low level of side-chain splitting enzyme in both testicular masses. Some 20β reduction was observed, the compound isolated being 4-pregnene-17α,20β-diol-3-one. No 11-oxygenated steroids were formed, perhaps because of the limited degree of 21-hydroxylation, as will be discussed.

When 17α-hydroxyprogesterone-4-C^{14} was used as a substrate, 80 to 88 per cent of the substrate was recovered unchanged (Table 2). Again, the results showed a low level of side-chain splitting enzyme activity. A very

TABLE 2. RESULTS OF INCUBATION OF 200 MG. OF HOMOGENIZED LEFT TESTICULAR TUMOR WITH PROGESTERONE-4-C^{14} AND 17α-HYDROXYPROGESTERONE-4-C^{14}

Steroids isolated (mμmoles)	Progesterone-4-C^{14} substrate		17α-OH-progesterone-4-C^{14} substrate	
	25 mμmoles	100 mμmoles	25 mμmoles	100 mμmoles
Material on origin	1.50	5.08	1.72	3.37
4-Pregnene-17α,20α-diol-3-one	2.58	8.92	1.11	3.44
4-Pregnene-17α,20β-diol-3-one	0.40	1.71	1.16	4.04
6β-Hydroxyprogesterone	0.43	1.78	—	—
17α-Hydroxyprogesterone	8.48	35.90	20.62	88.19
Unknown*	—	0.23	—	—
11-Deoxycorticosterone	0.13	0.72	—	—
Testosterone	0.06	0.34	—	—
4-Androstene-3,17-dione	0.16	0.91	0.39	0.96
4-Pregnene-20α-ol-3-one	0.11	0.78	—	—
Progesterone	10.79	42.24	—	—
Unknown†	0.36	1.39	—	—

* The free compound showed similar polarity to DOC and 17α-hydroxyprogesterone. Its acetate was more polar than DOCA.

† Less polar than pregnane-3,17-dione.

small amount of 4-androstenedione, but no detectable testosterone, was formed. The principal metabolites isolated were the corresponding 20α and 20β reduced compounds.

The large amount of 4-pregnene-17α,20α-diol-3-one formed from progesterone in contrast to the small amounts of that compound formed from 17α-hydroxyprogesterone indicates that the major sequence of reactions is as shown by the heavy arrows in Figure 1. Wiest (13) demonstrated that an enzyme isolated from ovaries reduces both progesterone and 17α-hydroxyprogesterone to the corresponding 20α-hydroxy compounds. However, 17α-hydroxyprogesterone seems to be a less reactive substrate than progesterone.

The results obtained when using 11-deoxycorticosterone-21-C^{14} as a substrate are summarized in Table 4. The criteria for identification of products are given in Table 3. A limited but significant amount of corticosterone and about the same amount of a more polar compound, finally identified as 6β-hydroxy-11-deoxycorticosterone, were formed.

TABLE 3. CRITERIA FOR IDENTIFICATION OF RADIOACTIVE STEROIDS [illegible]

TABLE 3. CRITERIA FOR IDENTIFICATION OF RADIOACTIVE STEROIDS ISOLATED IN THE *in vitro* STUDIES

Steroid isolated	Free form S.S.*	Acetylation S.S.	Oxidation† S.S.	Crystallization to constant specific activity‡
4-Pregnene-17α,20α-diol-3-one	§Bf → HBf	20α-monoacetate	HBf → {30% 17α-hydroxyprogesterone; 70% 4-androstene-3,17-dione}	→ Cr 67, 65, 62, 68; → Cr 128, 132, 125, 120
4-Pregnene-17α,20β-diol-3-one	Bf → §H_{8h}	20β-monoacetate	§H_{8h} → 4-androstene-3,17-dione	→ Cr 87, 88, 92, 87
6β-Hydroxyprogesterone	§Bf → H_{8h}	6β-monoacetate	H_{8h} → 6-keto-progesterone	Not crystallized
17α-Hydroxyprogesterone**	HBf → HBf	(remains free)	HBf, MC-PG, H_{48h} → (remains unchanged)	→ Cr 472, 485, 468, 462
11-Deoxycorticosterone	HBf → §HBf, H_{6h}	21-monoacetate	Bf → 3-keto-4-etiocholenic acid	Not crystallized; → Cr 63, 63, 52, 59
Testosterone	HBf → §HBf, Hf	17β-monoacetate	Hf → 4-androstene-3,17-dione	→ Cr 45, 48, 39
4-Androstene-3,17-dione**	HBf, Hf → Hf	(remains free)	Hf, H_{8h} → (remains unchanged)	→ Cr 246, 256, 238, 236
4-Pregnene-20α-ol-3-one	HBf, Hf → §Hf	20α-monoacetate	Hf → progesterone	→ Cr 62, 56, 58
Progesterone	HBf, Hf → §Hf	(remains free)		Not crystallized
Corticosterone**	Bf, B_{8hs} → HB_{4h}	21-monoacetate	HBf → (#) 11-dehydrocorticosterone-21-monoacetate	Not crystallized
6β-Hydroxy-DOC	Bf, B_{8hs} → §HBf	6,21-diacetate		Not crystallized
6β-Hydroxytestosterone	Cf → §Bf	6β,17β-diacetate	Bf → {60% 6β-OH-4-androstenedione; 40% 6-keto-4-androstenedione}	Not crystallized; Not crystallized
11β-Hydroxytestosterone	§B_{18h}			→ Cr 46, 40, 48
6β-Hydroxy-4-androstenedione	§B_{4h} → Bf	6β-monoacetate		Not crystallized
11β-Hydroxy-4-androstenedione	§B_{1h} → Bf	(remains free)		→ Cr 172, 173, 154, 166
			Bf → adrenosterone	→ Not crystallized
Adrenosterone	§Bf → HBf	(remains free)	H_{24h} → (unchanged)	Not crystallized
Androstanedione	H_{8h} → *Hf	(remains free)		Not crystallized

* S.S. denotes solvent systems used: H = Hexane-formamide; B = Benzene-formamide; HB = Hexane-benzene-formamide; C = Chloroform-formamide; MC-PG = Methylcyclohexane-propylene glycol.
Development of chromatograms: f = chromatograms developed to a given solvent front; 4h, 8h, etc. = time of dropping in hours for chromatograms which were overrun.
† The compounds formed after oxidation of the unknown and standards were obtained from either the previously saponified acetate or from aliquots of the free material, except for (#), in which case the acetate was directly oxidized.
‡ Specific activity is expressed in counts per minute per milligram for samples which were crystallized (Cr). Samples which were not crystallized are indicated.
§ Internal standard added after the first paper chromatography.
** Internal standard added to the crude extract.

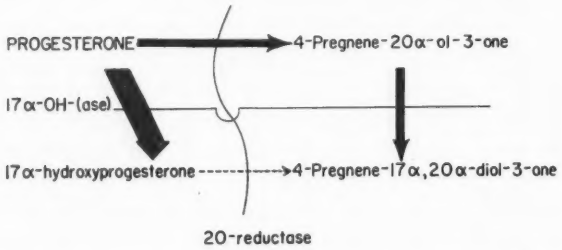

FIG. 1. Suggested biosynthetic pathways of 4-pregnene-17α,20α-diol-3-one in these testicular tumors. Where the light lines leading from the enzymes cross arrows, the action of this specific enzyme is indicated. Width of the arrows is proportionate to the particular enzyme activity.

When testosterone-4-C^{14} and 4-androstenedione-4-C^{14} were used as substrates for incubation, the products listed in Table 5 were obtained. Again, the criteria of identification are given in Table 3. The two steroids did not interconvert extensively in either direction. Two major enzyme reactions were observed in these incubations: 1) 6β-hydroxylation, accounting for 6β-hydroxy-4-androstenedione and for 6β-hydroxytestosterone, both of which were isolated and identified, and 2) 11β-hydroxylation. Even though the amounts of 11-oxysteroids formed were small, the high specific activity that was used enabled us to identify them as 11β-hydroxy-4-androstenedione (from 4-androstenedione), and 11β-hydroxytestosterone (from testosterone); we were also able to identify adrenosterone from both these substrates.

Some radioactive metabolites, isolated but not identified, are described in footnotes to Tables 2, 4 and 5.

The results of chromatographic analysis of chloroform extracts from spermatic vein plasma are shown in Figure 2. Neither 4-androstenedione nor 11β-hydroxy-4-androstenedione was detectable in the samples. All of the samples showed a UV spot (zone A) slightly more polar than 11β-hydroxy-4-androstenedione. These areas were combined after elution and

TABLE 4. RESULTS OF INCUBATION OF 200 MG. OF HOMOGENIZED LEFT TESTICULAR TUMOR WITH 11-DEOXYCORTICOSTERONE-21-C^{14} (DOC)

Steroids isolated (mμmoles)	DOC-21-C^{14} substrate (25 mμmoles)
Material on origin	11.57*
6β-Hydroxy-DOC	1.40
Corticosterone	1.49
Unknown†	2.81
11-Deoxycorticosterone	7.73

* The more polar material was not completely analyzed.
† Slightly more polar than DOC. This compound forms an acetate.

TABLE 5. RESULTS OF INCUBATION OF 200 MG. OF HOMOGENIZED LEFT TESTICULAR TUMOR WITH TESTOSTERONE-4-C^{14} AND 4-ANDROSTENE-3,17-DIONE-4-C^{14}

Steroids isolated (mμmoles)	4-Androstenedione-4-C^{14} substrate		Testosterone-4-C^{14} substrate	
	25 mμmoles	100 mμmoles	25 mμmoles	100 mμmoles
Material on origin	0.54	1.65	0.73	1.27
6β-Hydroxytestosterone	—	—	0.74	2.24
Unknown*	0.16	0.82	—	—
11β-Hydroxytestosterone	—	—	—	0.04
6β-Hydroxy-4-androstenedione	0.27	1.11	—	—
Unknown†	—	—	0.40	1.00
11β-Hydroxy-4-androstenedione	0.45	1.89	—	—
Unknown‡	—	—	0.54	1.83
Adrenosterone	0.13	0.53	—	1.45
Testosterone	0.66	2.81	21.33	90.31
4-Androstene-3,17-dione	22.50	90.44	1.26	1.86
Androstanedione	0.10	0.22	—	—
Unknown§	0.19	0.54	—	—

* Moved like 19-hydroxy-4-androstenedione.

† Moved like 6β-hydroxy-4-androstenedione. The acetates also behaved similarly but after chromic acid oxidation of the free compounds, the radioactivity did not coincide with the UV position of 6-keto-4-androstenedione.

‡ Less polar than 11β-hydroxy-4-androstenedione. Formed a monoacetate. 11-Keto-testosterone and 6-keto-testosterone were eliminated as possibilities.

§ Slightly more polar than androstanedione.

rechromatographed on paper. The spots behaved identically with standard 4-pregnene-17α,20α-diol-3-one.

Sample 1 (spermatic vein plasma collected immediately before administration of ACTH) did not show ultraviolet-absorbing material at the position of testosterone; however, samples 2 and 3, which had been collected after the administration of a total of 6.5 units and 7.5 units of ACTH respectively, exhibited dark ultraviolet spots in the testosterone position,

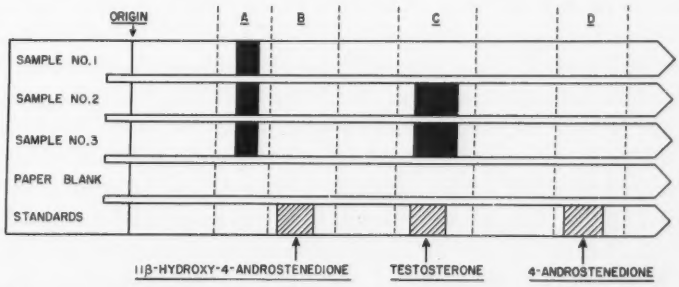

FIG. 2. Paper chromatographic analysis of chloroform extracts from spermatic vein blood. Sample 1—collected before treatment (control). Sample 2—collected after 6.5 units of ACTH injected intravenously. Sample 3—collected after 7.5 units of ACTH injected intravenously. Solvent system: hexane-formamide (defatting) and hexane-benzene-formamide (development).

the spot obtained from sample 2 being darker than that from sample 3. These C zones turned blue in the presence of I_2-KI, suggesting the presence of 17α-hydroxyprogesterone, which behaves identically with testosterone in the chromatographic system used. The yellow-brownish color that testosterone produces was not observed. Results of the Zimmermann test were negative for all of the 3 chromatographed samples, showing that 17-ketosteroids were not present in the spermatic vein blood.

The ultraviolet spectrum obtained with sample 2C exhibited a clear

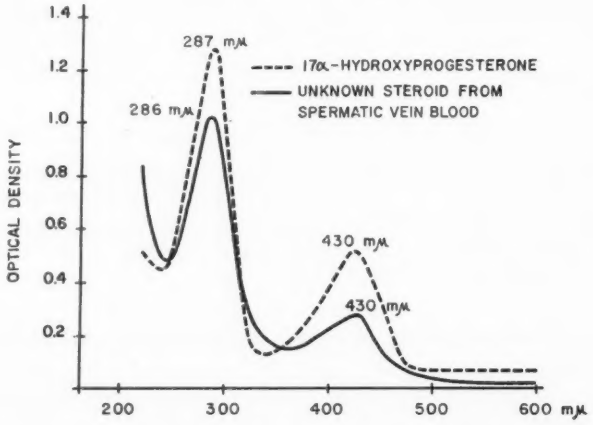

FIG. 3. Sulfuric acid spectra of 17α-hydroxyprogesterone and the steroid isolated from spermatic vein blood after administration of ACTH.

absorption maximum at 240 mμ, whereas the weaker sample 3C showed a symmetrical peak at 240 mμ. When the Allen (14) correction was applied, the absorbancies corresponded to 23 μg. and about 15 μg. of 17α-hydroxyprogesterone respectively. Correcting for the different volumes in the two samples, the concentration in plasma from spermatic vein blood was thus 330 μg. per 100 ml. for sample 2 and about 375 μg. per 100 ml. for sample 3.

The eluate from sample 2C and the corresponding eluate of the paper blank were then dried under nitrogen. Concentrated sulfuric acid (2 ml.) was added to the samples and the mixture was incubated for two hours at room temperature. The sulfuric acid spectrum of the unknown is shown in Figure 3, in comparison with the sulfuric acid spectrum developed with standard 17α-hydroxyprogesterone. The eluate of sample 3C was dried and acetylated with a mixture of acetic anhydride/pyridine (1:5, v/v) and the extract rechromatographed on a strip 1 cm. wide, in the hexane-benzene-formamide system in parallel with a standard mixture of 17α-hydroxy-

progesterone and testosterone acetate. The unknown UV spot again behaved like 17α-hydroxyprogesterone, which does not form an acetate under the conditions used.

DISCUSSION

The results of incubation of these testicular tumors indicate a higher ratio of 17α-hydroxyprogesterone to C_{19} steroids (4-androstenedione and testosterone) than occurs in normal testicular tissue. This finding appears to be due to the low activity of the side-chain splitting enzyme in the tumors. The tissue incubation studies are corroborated by the large amounts of 17α-hydroxyprogesterone and the absence of detectable 4-androstenedione and testosterone in the spermatic vein blood.

The finding of 11β-hydroxylase activity is of particular interest. No 11β-hydroxylase activity could be demonstrated in rodent testes in previous investigations. This is not conclusive, however. Experiments in our laboratory (7) have shown that normal rat and mouse testes possess 21-hydroxylase activity, which can be observed when carefully sought, although previously this enzyme was considered to be limited to the adrenal cortex. As far as we are aware, no 11β-hydroxylase activity has been observed in normal human testes, but this requires further study. Savard *et al.* (15) found 11β-hydroxylase activity in a human testicular tumor, as indicated by the formation of 11β-hydroxy-19-carbon steroids from testosterone by the tumor tissue *in vitro*. They considered the possibility that the tumor arose from adrenocortical rest cells.

These authors suggested that two different 11β-hydroxylases may exist, one using C_{19} steroids as substrates, the other using C_{21} steroids. The concept arose from their finding that when testosterone was incubated with the tumor, three different 11β-oxysteroids were formed, but when progesterone-4-C^{14} was incubated, no 11β-hydroxylation occurred. Similarly, in the present case we demonstrated conversion of both testosterone and 4-androstenedione to 11-oxysteroids, but could not find formation of 11-oxysteroids from progesterone; however, when 11-deoxycorticosterone was used as substrate, small but significant amounts of corticosterone were identified, indicating that 11-hydroxylation of C_{21} steroids could occur. This is indicated in Tables 4 and 5. In the incubations with 25 mμmoles of substrate the 11β-hydroxylase used approximately three times as much 11-deoxycorticosterone (yielding 1.49 mμmoles of corticosterone) as 4-androstenedione (yielding 0.45 mμmole of 11β-hydroxy-4-androstenedione). It seems probable that the results observed by Savard *et al.* were due to the importance of 21-hydroxylation in increasing the binding of C_{21} steroids to the 11β-hydroxylase (16) rather than to the lack of a specific 11β-hydroxylase for these steroids.

The tumor tissue did not synthesize 17-ketosteroids in amounts adequate to explain the high levels of 17-ketosteroids excreted in the urine. It is likely that part of the large quantity of 17α-hydroxyprogesterone produced by the tumor *in vivo* was being transformed into 17-ketosteroids elsewhere, probably by the liver. The decrease in 17-ketosteroid excretion after mono-orchidectomy to 79 mg. per day, and after bilateral orchidectomy to 13.5 mg. per day, favors this hypothesis. An increase in the excretion of 17-ketosteroids after administration of 17α-hydroxyprogesterone has been found in animals (17) and in human beings (18–20).

The 24-hour excretion of pregnanetriol, which was as high as 66.2 mg. before operation, dropped to 15.4 mg. after unilateral, and to 7.4 mg. after bilateral orchidectomy. This confirms the evidence for a high level of secretion of 17α-hydroxyprogesterone by the tumors, and indicates that only a portion of the original secretory product underwent side-chain splitting to 17-ketosteroids.

An interesting observation is that these testicular tumors responded to administration of ACTH by increasing the output of 17α-hydroxyprogesterone, undetectable in spermatic vein blood before ACTH but present in high concentration after stimulation. Also, urinary excretion of 17-ketosteroids and pregnanetriol was suppressed by 9α-fluorohydrocortisone (1). These findings resemble those of Bongiovanni and his co-workers in congenital adrenal hyperplasia, and also for the products formed on incubation of tissue (in so far as they identified them), the compounds secreted into the blood, and the metabolites excreted in the urine (21, 22). We therefore conclude that these testicular tumors resemble the original abnormal adrenal tissue. They probably arise from bilateral adrenal rests, since interstitial cells have never been shown to respond to adrenocorticotropin.

Acknowledgments

I should like to express to Dr. Edgar J. Schoen and Dr. Vincent Di Raimondo my appreciation of the opportunity to perform the *in vitro* experiments described. The technical assistance of Mrs. Barbara Samuels, Miss Marian Aucutt, and Miss Margot Bartko is appreciated.

The steroids used as standards for the identification of the metabolites formed in the *in vitro* studies are generally available, with the following exceptions: 4-pregnene-17α, 20α-diol-3-one and 4-pregnene-17α,20β-diol-3-one, which were generously supplied by Dr. Thomas F. Gallagher; and 4-pregnene-20α-ol-3-one, supplied by Dr. Walter G. Wiest. I express my gratitude for their collaboration.

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JCEM

URINARY HORMONE STUDIES IN UNTREATED CONGENITAL ADRENAL HYPERPLASIA DURING AND AFTER PREGNANCY

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ABSTRACT

A 22-year-old patient is described with congenital adrenal hyperplasia and spontaneous menstrual cycles who became pregnant prior to the institution of steroid therapy. During the latter part of gestation there was a progressive decrease in the daily urinary excretion of neutral 17-ketosteroids, pregnanediol and pregnanetriol. Excretion of the latter two compounds remained low until the sixteenth week, when spontaneous abortion occurred. Chromatographic separation of the urinary neutral 17-ketosteroids revealed a predominance of androsterone. Following the administration of corticotropin during pregnancy there was a pronounced increase in the urinary output of androsterone, a decrease in the output of etiocholanolone, and a rise in the ratio of androsterone to etiocholanolone from 1.3 to 5.8. These changes took place at a time when there was no significant urinary excretion of pregnanediol and pregnanetriol. The data indicate an alteration in either the production or metabolism of progesterone and 17α-hydroxyprogesterone, and suggest that the major source of the increased androsterone was an excess adrenal production of C_{19} steroids relative to C_{21} compounds. The presence of ovulatory menses in this patient demonstrates that the increased circulating androgens could not disrupt the normal pituitary-ovarian relationship despite their virilizing capabilities.

INTRODUCTION

CONGENITAL adrenocortical hyperplasia is due to impairment of the synthesis of cortisol by the adrenal cortex (1, 2). The abnormal adrenocortical function generally begins during the first trimester of intrauterine life and gives rise to pseudohermaphrodism in the female. Progressive virilization subsequently occurs, leading to a stunted and infertile adult. The administration of glucogenic steroids to such patients results in suppression of adrenocortical function with the eventual onset of ovulatory

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menses (3). Several patients thus treated have been delivered of normal full-term babies (4, 5).

Rarely, the adrenogenital syndrome may not become manifest until puberty or later (6–8). However, this group demonstrates defects in steroidogenesis identical with those observed in the prepuberal patients, as reflected by an increase in the urinary titers of pregnanetriol and neutral 17-ketosteroids. On occasion, the menarche may occur spontaneously and menstruation may continue more or less regularly for several years.

The present case represents an example of congenital adrenal hyperplasia with spontaneous regular menstrual cycles and constitutes the first reported instance in which pregnancy occurred in the untreated patient.

METHODS

The 24-hour urinary excretion of total neutral 17-ketosteroids was estimated by the colorimetric method of Holtorff and Koch (9). The 3β neutral 17-ketosteroids were precipitated with digitonin (10). The remaining 3α neutral 17-ketosteroids[1] were separated by paper chromatography using the Rubin modification of the Zaffaroni system (11), and identified by the Zimmermann color reaction (12). The solvent system was heptane saturated with propylene glycol. The perbenzoic acid oxidation of $\Delta^{9(11)}$ unsaturated compounds was omitted. This resulted in slightly increased values for androsterone and etiocholanolone. The second chromatogram was made up of three zones: 11-ketoandrosterone, 11-ketoetiocholanolone, and a mixture of the more polar components. The latter consisted of 11-hydroxylated 17-ketosteroids and an unresolved residual fraction. Pregnanediol and pregnanetriol were determined by the methods of Eberlein and Bongiovanni (13) and Bongiovanni and Eberlein (14) respectively. The urinary and blood levels of 17,21-dihydroxycorticoids were measured by the Peterson modification of the Silber-Porter method (15).

CASE REPORT

V.A., a 22-year-old married Negro female was referred to the Endocrine Clinic of the Jewish Hospital of Brooklyn in November 1959 for evaluation of hirsutism and enlargement of the clitoris, present since she was 8 years of age. Menses began at the age of 12, recurring every twenty-four to twenty-eight days and lasting four to six days. One year prior to admission a miscarriage had occurred after approximately six weeks of gestation.

On physical examination in November 1959 the patient was a well developed, normally proportioned female. The height was 145 cm. with a segmental ratio of 1.0; the weight was 102 pounds, and the blood pressure was 120/80 mm. Hg. A light hairy fuzz was present on the upper lip, chin and sides of the face; axillary hair was abundant. No breast tissue was palpable, although the nipples were erectile. Pubic hair was also abundant and of male configuration. The clitoris was 5.5 cm. long and 2.5 cm. in diameter (Fig. 1). The labia were unfused; the urethra and vagina were in their respective positions and normally separated. Pelvic examination revealed no abnormalities. Numerous cornified cells were present in the vaginal smear.

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[1] We are indebted to Dr. D. K. Fukushima of the Sloan-Kettering Institute for Cancer Research, New York, N. Y., for supplying several of the 17-ketosteroid standards.

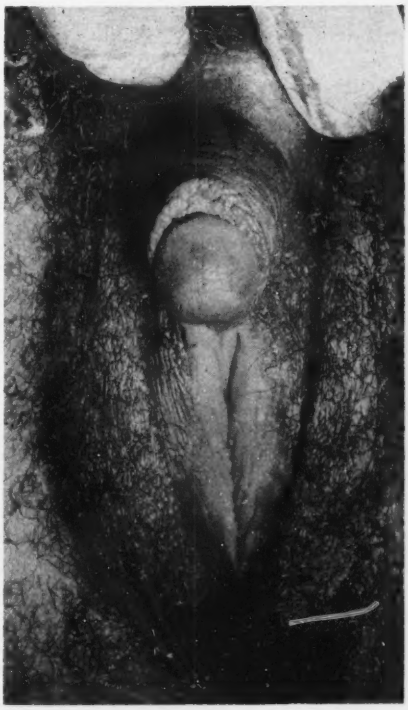

FIG. 1. *Mrs. V.A.* The external genitalia, showing the marked clitoral enlargement.

A skin biopsy showed chromatin-positive nuclei in the cells. The urinary excretion of the neutral 17-ketosteroids was 41.6 and 46.2 mg. per twenty-four hours (Table 1). Following the intravenous administration of 25 mg. of corticotropin (ACTH) over an eight-hour period, the 24-hour excretion of neutral 17-ketosteroids increased to 80 mg. A cortisone suppression test (Table 1) over a five-day period reresulted in a fall in the 24-hour excretion of neutral 17-ketosteroids to 6.2 mg. Urinary gonadotropin titers were positive at 10 and negative at 20 mouse uterine units. An intravenous pyelogram and perirenal gas insufflation failed to reveal any gross adrenal abnormalities.

The menstrual period due the latter part of January and that due in late February 1960 failed to appear. In March 1960 the patient noted sensitivity of both breasts. Physical examination disclosed a tender nodule of glandular tissue under each nipple. The uterus was globular, soft, and enlarged to the size of a 6- to 8-week pregnancy. Results of the Aschheim-Zondek test were positive. Cortisone was not administered until approximately the fourteenth week of gestation. Despite a prompt decrease in the urinary excretion of neutral 17-ketosteroids the patient spontaneously aborted a well formed male fetus two weeks after institution of the therapy (Fig. 2). Administration of cortisone was discontinued and four weeks later there was a return of normal menstrual cycles.

URINARY HORMONE EXCRETION DURING PREGNANCY

The data are listed in Table 2. During the second week there was an initial rise in the 24-hour urinary excretion of neutral 17-ketosteroids to 55.5 mg. followed by a progressive fall to a value of 27.6 mg. by the fourteenth week. The corresponding levels of urinary 17-hydroxycorticoids were normal throughout the period of study. Urinary pregnanediol and pregnanetriol values increased sharply to 49.9 and 25.4 mg. per twenty-four hours respectively during the eighth week of gestation, but there was a subsequent diminution to less than 1 mg. toward the end of the first tri-

TABLE 1. URINARY EXCRETION OF TOTAL NEUTRAL 17-KETOSTEROIDS BEFORE PREGNANCY; RESPONSE TO ACTH AND CORTISONE

Day of study	Medication	Urinary total neutral 17-KS (mg./24 hrs.)
Control period	None	41.6, 46.2
	25 units ACTH i.v. over 8 hrs.	80.0
1	Cortisone, 100 mg. daily (oral)	—
2	Cortisone, 100 mg. daily (oral)	31.0
3	Cortisone, 100 mg. daily (oral)	16.8
4	Cortisone, 100 mg. daily (oral)	7.9
6	Cortisone discontinued	6.2
14	None	26.8
21	None	40.0

FIG. 2. Well formed male fetus approximately 16 weeks of age. Testicular tissue was identified histologically.

mester. The excretion of these two compounds remained low until the abortion occurred in the sixteenth week.

Values for the individual fractions of the urinary total 17-ketosteroids are shown in Table 3. The proportion of beta 17-ketosteroids relative to the total was within the normal range. There was an increased output of both the 11-oxygenated and 11-deoxy 17-ketosteroids. As pregnancy advanced, the former declined while the latter tended to increase. The ratio of androsterone to etiocholanolone varied between 1.3 and 1.5.

TABLE 2. URINARY EXCRETION OF 17-KETOSTEROIDS, 17-OH-CORTICOSTEROIDS, PREGNANEDIOL AND PREGNANETRIOL DURING AND AFTER PREGNANCY: RESPONSE TO ACTH

Gestation (wks.)	Medication	Urinary steroid excretion (mg./24 hrs.)			
		Total 17-KS	17-OH-CS	Pregnanediol	Pregnanetriol
Control period	None	41.0	2.5	—	—
1	None	40.6	—	—	—
2	None	55.5	—	0.6	19.6
4	None	51.8	2.4	—	—
	25 units ACTH i.v. over 8 hrs.	81.5	2.6	—	—
8	None	38.4	2.3	49.9	25.4
9	None	36.8	—	—	11.4
11	None	33.5	3.0	—	—
12	None	33.9	3.0	0.6	0.3
14	None	27.6	2.1	0.9	0.3
	25 units ACTH i.v. over 8 hrs.	55.5	3.7	0.0	0.0
16		*Spontaneous Abortion*			
6 wks. after abortion	None	35.4	2.2	—	10.0
	25 units ACTH i.v. over 8 hrs.	61.5	3.6	—	31.0

Effect of ACTH

The intravenous administration of ACTH over an eight-hour period during the fourteenth week of pregnancy produced a twofold increase in the urinary excretion of neutral 17-ketosteroids without any significant increase in that of the 17-hydroxycorticoids, pregnanediol or pregnanetriol. A greater percentage increase was observed in the 11-oxygenated than in the 11-deoxy 17-ketosteroids. The urinary excretion of androsterone and 11-ketoetiocholanolone increased considerably, and the androsterone: etiocholanolone ratio rose from 1.3 to 5.8. This occurred at a time when

TABLE 3. URINARY 17-KETOSTEROID EXCRETION PATTERNS DURING AND AFTER PREGNANCY: RESPONSE TO ACTH

Gestation (wks.)	Medication	Total neutral 17-KS (mg./24 hrs.)	17-Ketosteroid fractions [mg./24 hrs. and (%) of total 17-KS]								
			Beta fraction	Alpha fraction							
				Andro.	Etio.	Andro./ Etio. ratio	Total 11-deoxy 17-KS	11-Keto-andro.	11-Keto-etio.	More polar components	Total 11-oxy 17-KS
8	None	38.4	2.2 (5.7)	8.5 (22.1)	5.8 (15.1)	1.5	16.5 (43.0)	3.7 (9.7)	3.1 (8.1)	3.3 (12.6)	10.1 (26.4)
11	None	33.5	6.8 (20.3)	8.3 (24.5)	5.7 (16.9)	1.5	20.7 (61.8)	2.8 (8.4)	3.1 (9.3)	3.1 (9.3)	9.0 (26.9)
12	None	33.9	5.5 (16.1)	7.0 (20.6)	5.1 (15.0)	1.4	17.6 (52.0)	1.6 (4.7)	1.8 (5.3)	3.6 (10.6)	7.0 (20.6)
14	None	27.6	4.2 (15.2)	7.8 (28.3)	6.2 (22.4)	1.3	18.2 (66.0)	1.0 (3.6)	0.8 (2.9)	2.1 (7.6)	3.9 (14.1)
	25 Units ACTH i.v. over 8 hrs.	55.5	2.8 (5.1)	21.4 (38.6)	3.7 (6.7)	5.8	27.9 (50.1)	4.1 (7.4)	5.9 (10.6)	6.7 (12.1)	16.7 (30.1)
16	*Spontaneous Abortion*										
6 wks. after abortion	None	31.1	7.4	10.7 (34.4)	9.9 (31.8)	1.1	22.9 (73.5)	3.2 (10.2)	2.6 (8.4)	3.4 (10.8)	9.2 (29.6)
	25 Units ACTH i.v. over 8 hrs.	61.1	5.7	22.4 (36.8)	13.6 (22.2)	1.7	39.5 (64.5)	3.7 (6.1)	5.2 (8.5)	4.0 (6.5)	12.9 (21.2)

there was no measurable urinary pregnanetriol and presumably no significant production of its precursor, 17α-hydroxyprogesterone. The beta fraction decreased relative to the total 17-ketosteroids.

URINARY HORMONE EXCRETION AFTER SPONTANEOUS ABORTION

Six weeks after the abortion, the 24-hour urinary excretion of neutral 17-ketosteroids was 31.1 mg., and that of 17-hydroxycorticoids was normal. The beta fraction was within normal limits, but the alpha fractions were generally increased. The pregnanetriol output was 10.0 mg. per twenty-four hours (Table 2).

The intravenous administration of ACTH over an eight-hour period produced a twofold increase in the urinary excretion of neutral 17-ketosteroids, but the plasma 17-hydroxycorticoid levels rose only slightly—from 10 μg. to 14 μg. per 100 ml. There was no significant change in the urinary excretion of 17-hydroxycorticoids. In addition, the urinary output of pregnanetriol increased threefold, with an approximately parallel increase in the output of 11-oxygenated and 11-deoxy 17-ketosteroids. The androsterone:etiocholanolone ratio was 1.1 before administration of ACTH, and 1.7 directly afterward (Table 3).

DISCUSSION

The diagnosis of congenital adrenal hyperplasia in this patient was suggested by the history, the clinical manifestations and the laboratory data. In addition, the inadequate rise in the levels of plasma and urinary 17-hydroxycorticoids and the marked increase in the urinary excretion of pregnanetriol after the administration of ACTH indicated a defect in the adrenal elaboration of hydrocortisone.

Chromatographic separation of the urinary neutral 17-ketosteroids revealed a predominance of androsterone. Following the administration of ACTH during pregnancy there was a marked increase in the urinary output of androsterone, a decrease in the excretion of etiocholanolone, and a rise in the androsterone:etiocholanolone ratio from 1.3 to 5.8 (Table 3). These changes were noted at a time when there was virtually no urinary excretion of pregnanediol and pregnanetriol (Table 2). These data indicate that progesterone and 17α-hydroxyprogesterone were not being produced in significant quantities, or that they were being metabolized along an alternate pathway to end-products not measured by our method. Thus the results suggest that the major source of the increased androsterone was an excess adrenal production of C_{19} steroids relative to C_{21} compounds. This may have involved a direct conversion of cholesterol to dehydroepiandrosterone, or possibly interconversion through a C_{21} intermediate such as pregnenolone and subsequently to dehydroepiandrosterone (16). It is of

interest that the decreased excretion of progesterone metabolites in our patient during the latter part of the first trimester is similar to that observed in normal pregnant women during a comparable period (17).

The congenital adrenogenital syndrome is generally associated with ovarian failure. The ovarian failure is presumably related to the increased levels of circulating androgens and estrogens, thus accounting for the reduction in the pituitary elaboration of gonadotropins (18). In our patient the increased circulating levels of androgens were sufficient to produce virilization, but were incapable of disrupting the normal pituitary-ovarian relationship, as shown by the onset of pregnancy.

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JCEM

PLASMA 17-HYDROXYCORTICOSTEROIDS IN CUSHING'S SYNDROME*

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ABSTRACT

Plasma levels of 17-hydroxycorticosteroids (17-OH-CS) during the night and day were studied in 22 cases of Cushing's syndrome. The normal diurnal variation was lacking in most of these patients; instead, the night levels were higher than normal. The mean concentration of plasma 17-OH-CS often differed markedly between different days, but the absence of diurnal variation persisted even in the presence of a lower level of plasma steroids. In 3 subjects, however, there was a decrease in steroid levels during the night; in 2, the values approached the normal range. Data are presented on a few patients with a monosymptomatic clinical picture and normal urinary excretion of 17-ketogenic steroids. Theoretical and diagnostic implications based on the absence of variation in plasma 17-OH-CS concentration are discussed.

INTRODUCTION

THE clinical manifestations of Cushing's syndrome are mainly if not wholly explained by the effects of an abnormally increased secretion of cortisol. Measurement of the daily urinary excretion of the metabolites of cortisol and related steroids is a generally accepted method for the laboratory confirmation of the clinical diagnosis of Cushing's syndrome. The determination of plasma cortisol concentration is now a feasible method for routine use in a steroid laboratory, especially since the development of accurate methods not involving chromatographic separations (1). The significance of the level of plasma cortisol for the diagnosis of Cushing's syndrome, however, has not yet been fully explored.

It is well established that normally a marked diurnal variation occurs in the plasma level of 17-hydroxycorticosteroids (17-OH-CS), the peak values being found early in the morning and the lowest during the night

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(2–3). Lindsay *et al.* (4) in 1956 found a high plasma 17-OH-CS level during the day and night in 3 patients suffering from Cushing's syndrome, and recently Doe *et al.* (5) demonstrated a lack of variation in plasma and urinary 17-OH-CS in 5 patients with Cushing's syndrome.

This report presents data on plasma levels of 17-OH-CS during the day and night in 22 subjects with Cushing's syndrome. The constant levels of corticosteroids are confirmed, and these levels are compared to other steroid data and to the clinical picture. The significance of these findings for the diagnosis of Cushing's syndrome is discussed.

MATERIAL AND METHODS

Patients

The group comprised 21 patients with Cushing's syndrome and 1 woman with an apparently complete remission of Cushing's syndrome after partial adrenalectomy performed five years before the present study (Case 22). Data on sex, age and duration of symptoms are presented in Table 1. All subjects were operated upon except Patient No. 18, who three years before this study had received therapeutic irradiation of the pituitary which was followed by an incomplete remission. It should be emphasized that the diagnosis of Cushing's syndrome in our series did not depend upon the results obtained from measurements of 17-OH-CS in plasma. These measurements were made in cases in which the diagnosis had already been ascertained by accepted clinical and laboratory studies not including the measurement of plasma cortisol.

Hypophysectomy in Case 14 was followed by complete remission of her severe illness and psychosis. In Case 1, an adrenocortical cancer was removed, and in Cases 10 and 21 an adenoma was extirpated. In the other patients, bilateral adrenalectomy was performed; the adrenal cortices were found to be hyperplastic or morphologically normal in 2 (Cases 12, 19). In most cases a postoperative observation period of six months or more allowed retrospective confirmation of the diagnosis through the definite amelioration or disappearance of pathologic signs and symptoms. In Cases 11, 20 and 21, however, sufficient time had not elapsed after operation to permit a significant degree of regression. Four of these cases (Nos. 18, 19, 20 and 21) presented diagnostic difficulties because of an unusual clinical picture and failure to demonstrate an increased urinary excretion of cortisol metabolites. In these 4 cases the rationale of the diagnosis will be discussed in some detail.

Methods

17-Hydroxycorticosteroids (17-OH-CS) in plasma were assayed in accordance with the method of Eik-Nes (1). In this report, the term plasma 17-OH-CS will be used synonymously with (free) cortisol.

17-Ketogenic steroids (17-KGS) in urine were determined according to the procedure of Appleby *et al.* (6).

17-Ketosteroids in urine were estimated by the Zimmermann reaction.

Administration of triamcinolone. To demonstrate the well known resistance of cortisol secretion to feed-back steroid inhibition in some patients, triamcinolone (9α-fluoro-11β,16α,17,21-tetrahydroxy-pregna-1,4-diene-3,20-dione) was used in a dosage of 4–8 mg. every six hours.

TABLE 1. URINARY EXCRETION OF 17-KETOGENIC STEROIDS (17-KGS; WITHIN PARENTHESES ARE THE NUMBER OF OBSERVATIONS) AND 17-KETOSTEROIDS (17-KS), AS WELL AS THE PLASMA CONCENTRATION OF 17-HYDROXYCORTICOSTEROIDS (17-OH-CS) DURING THE DAY AND NIGHT IN 22 CASES OF CUSHING'S SYNDROME

Case No.	Initials	Sex	Age (yrs.)	Duration of symptoms (yrs.)	17-KGS mean (mg.)	17-KGS range (mg.)	17-KS mean (mg.)	17-KS range (mg.)	Plasma 17-OHCS (μg./100 ml.) 00 04 08 12 16 20 24 Hours
1.	M.W.	F	52	>1/2	83(15)	55-112	35	17-53	19 21 32 31 29 23 21 24
2.	B.Q.	M	36	>2	83(10)	46-123	55	35-63	54 52 40 19 14 12
3.	C.H.	M	21	>2	46(10)	26-64	22	13-35	31 22 28 24 33 29 24 30
4.	W.S.	M	40	>2	45(6)	36-51	26	23-27	18 23 21 19 20 14 21 18
5.	J.E.	F	35	>1	42(14)	28-48	34	16-56	20 24 22 24 15 27 28 13
6.	G.L.	M	41	10?	41(9)	34-54	30	17-44	19 21 16 16 17 15 17 19
7.	I.P.	F	37	>5	40(6)	32-49	20	15-29	18 18 14 11
8.	R.O.	F	23	>2	39(7)	25-59	29	20-47	26 30 22 21 18
9.	M.A.	F	37	>11/2	39(9)	29-47	19	14-27	18 20 15 23 11 8
10.	K.P.	F	37	>1	38(8)	28-48	9	6-12	21 22 25 28 24 23 26 21
11.	S.P.	F	45	>1	38(8)	28-46	13	11-17	20 13 21 8 15 16
12.	G.L.	F	31	>1	29(15)	20-37	18	12-28	19 22 19 17 14 14 13 19
13.	E.A.	F	36	>1	28(5)	26-30	28	23-30	19 20 17 14 11 18
14.	S.N.	F	53	>5	27(6)	16-36	43	30-58	22 30 21 24 17 24
15.	G.J.	F	39	>2	26(16)	9-38	14	8-27	19 20 24 24 25 19 21 22
16.	U.D.	F	17	>2	25(17)	14-38	22	13-32	21 15 21 20 18 15 17 20
17.	G.R.	F	37	>3	22(15)	8-33	30	23-36	14 16 16 16 16 13
18.	A.O.	F	36	>5	16(8)	11-27	17	12-21	9 19 18 13 19 14
19.	G.S.	F	37	>5	15(9)	11-21	15	10-23	17 14 17 12 14 12 17 19
20.	A.D.	F	51	>2	15(8)	9-24	8	4-11	14 15 10 12 12 12 11 14
21.	I.M.	F	41	>8	15(13)	7-19	8	6-9	18 13 16 16 16 16 19 15
22.	L.L.	F	55	>10	6(2)	6;6;	4	3-5	11 11 8 6 6 8

RESULTS

In Table 1, one series of plasma levels of 17-OH-CS during the day and night is presented for each subject, as well as data on the urinary excretion of 17-KS and 17-KGS. The patients are arranged according to the mean excretion of 17-KGS. In Case 22 the values were obtained during clinical remission after partial adrenalectomy; they will be considered separately under Discussion. In 9 subjects, two or more series of data on the diurnal variation of 17-OH-CS were collected. These observations are presented in Table 2.

In most series the plasma 17-OH-CS concentration was almost constant but in others it varied. However, this variability was irregular, and in 4 patients only (Nos. 9, 11, 17 and 18) were single determinations of plasma 17-OH-CS below the level of 10 μg. per 100 ml.

In some subjects the plasma concentration of free cortisol varied appreciably between different days, but the absence of diurnal variation persisted even in the presence of a lower mean level of plasma cortisol. There were a few exceptions, however. In Case 2 the high concentration of plasma cortisol during the morning hours decreased markedly in the afternoon, reaching a level of 12 μg. per 100 ml. at midnight. A decrease in plasma 17-OH-CS concentration to 8 μg. per 100 ml. was also found during the night in Case 9. In Case 17 plasma cortisol was studied on five different occasions during the course of eighteen months; the levels, which were relatively low, did not show much variation but were lowest during the night in 3 of the series (Table 2). It may be seen from Tables 1 and 2 that in many cases the morning concentration of plasma 17-OH-CS was within the normal range (Table 4).

As expected, the urinary excretion of 17-KGS was increased in most subjects. In a few (Nos. 18, 19, 20 and 21), however, most or all single measurements were within the normal range. Table 1 shows that there was in many patients a marked variation in the urinary excretion of 17-KGS. The excretion of 17-KS generally parallelled the pattern of the 17-KGS. In patients with an adenoma (Nos. 10 and 21) as well as in Patient No. 20, the 17-KS were within the normal range.

In Table 3 the effects of triamcinolone on the urinary steroids and plasma 17-OH-CS in Cases 18, 19, 20 and 21 are shown. In Cases 20 and 21 the absence of significant adrenal inhibition is evident. In Cases 18 and 19 there was a significant decrease in the urinary excretion of 17-KS as well as of 17-KGS. In Case 18, however, the patient was receiving a dose of triamcinolone corresponding to 200 mg. of cortisone daily, and probably adrenal inhibition was incomplete, as on the sixth day of treatment the excretion of 17-KGS was only 9 mg. (mean basal level, 16 mg.) and repeated measurements failed to demonstrate plasma 17-OH-CS levels lower

TABLE 2. PLASMA LEVELS OF 17-HYDROXYCORTICOSTEROIDS IN 9 PATIENTS WITH CUSHING'S SYNDROME. FOR EACH PATIENT, TWO OR MORE SERIES OF MEASUREMENTS DURING THE DAY AND NIGHT ARE LISTED

Case No.	Day of study	Plasma 17-OHCS (μg./100 ml.) 00	04	08	12	16	20	24 Hours
1.	4/13/60	19	21	32	31	29	33	21	24
	5/11/60	26	28	35	31	29	34	27	31
3.	11/25/59	31	22	28	24	33	29	24	30
	1/10/60	16	14	14	14	17	17	14	14
6.	11/11/59	19	21	16	16	17	15	17	19
	11/18/59			13	19	21	18		17
9.	12/8/59	18		20	15	23	11		8
	1/14/60	34		38	31	31	31		31
11.	6/17/60	20	13	21	8	15		16	
	7/4/60	21	9	15	9	16		10	
16.	2/5/60	21	15	21	20	18	15	17	20
	2/24/60	34	27	26	26	24	24	30	24
17.	3/5/58			15	7	13	13		
	12/8/58	13		13	12	10		10	9
	1/7/59	10		13	12	13		10	8
	9/16/59	8	12	11	9	9	12	21	6
	9/30/59	14	16	16	16	16	13		
20.	6/2/60	14	15	10	12	12	12	11	14
	6/9/60	29	28	26	24	25	31	27	23
21.	10/14/58		20	20	18	17			
	2/16/59	18	15	16	15	17	13	16	15
	9/23/59	18	13	16	16	16	16	19	15
	3/23/60		19	15	17	17	18	17	16

than 10 μg. per 100 ml. The interpretation of the inhibition test in Case 19 is conjectural and it cannot be stated with certainty whether or not this patient showed an abnormal response to the given dose of triamcinolone.

In Table 4 are shown the concentrations of plasma 17-OH-CS in 23 hospitalized subjects with diverse diseases exclusive of endocrine, neuro-

TABLE 3. URINARY EXCRETION OF 17-KETOSTEROIDS AND 17-KETOGENIC STEROIDS AS WELL AS THE MORNING PLASMA LEVEL OF 17-HYDROXYCORTICOSTEROIDS DURING ADMINISTRATION OF TRIAMCINOLONE IN 5 CASES OF CUSHING'S SYNDROME

Case No.	Treatment	Urine		Plasma 17-OH-CS (μg./100 ml.)
		17-KS (mg./24 hrs.)	17-KGS (mg./24 hrs.)	
18	Day 6—triamcinolone (32 mg./24 hrs.)	4	9	10
19	Day 4—triamcinolone (16 mg./24 hrs.)	7	3	9
20	Day 4—triamcinolone (32 mg./24 hrs.)	11	21	28
21	Day 10—triamcinolone (32 mg./24 hrs.)	6	21	15

logic or psychiatric disorders. Blood samples were withdrawn at eight o'clock in the morning and at midnight. The patients were in a good general condition, without disturbances in sleep patterns.

DISCUSSION

That diurnal variation of adrenal activity exists has been known since the studies of Pincus (7) and Tyler *et al.* (8). Extensive data on the variability of plasma 17-OH-CS were presented by Migeon *et al.* in 1956 (3). They reported that the lowest plasma levels were found between 10:00 and 2:00 a.m. and that the maximal levels were usually found at 6:00 a.m. These findings have been fully confirmed by others (9). Apparently, the first recognition of the absence of diurnal variation in Cushing's syndrome was that of Laidlaw *et al.* (10). The first specific data, however, concerning the plasma levels of cortisol during the night and day were presented 1956, when Lindsay *et al.* (4) showed in 3 patients (2 with an adrenal tumor and 1 with hyperplasia) that the levels of cortisol were not only high but also almost constant. Recently Doe *et al.* (5) demonstrated the same lack of variability in 5 cases of Cushing's syndrome.

It should be restated at this point that the diagnosis of Cushing's syndrome in our patients was made independently of the results of the measurements of plasma 17-OH-CS. In Cases 1–17 the clinical picture and laboratory findings were diagnostic and the postoperative time of observation was sufficient (except for Case 11) to permit symptoms to disappear.

TABLE 4. MORNING AND MIDNIGHT LEVELS OF PLASMA 17-HYDROXYCORTICOSTEROIDS IN 23 SUBJECTS WITHOUT ENDOCRINE DISEASE

Time	Plasma 17-OH-CS (μg./100 ml.)
8 a.m.	14.3 ± 4.3* (8.6–27.8)**
12 midnight	3.5 ± 1.8 (1.0– 6.6)

* Standard deviation.
** Range.

The rationale of the diagnosis in Cases 18–21 will be discussed in some detail because the patients presented with few symptoms or atypical clinical pictures. Cases 1–21 comprise all cases of Cushing's syndrome studied in our hospital from January 1958 to October 1960, and no case was excluded from this series because of the finding of a normal variation of plasma cortisol.

It is apparent from Tables 1 and 2 that among the laboratory data presented, high night levels of plasma cortisol were the most uniform abnormal findings. In most patients, the normal variation of free plasma cortisol was lacking, whenever tested. In a few subjects (Nos. 2, 9 and 17), however, there was a definite decrease in night values. The marked differences between the mean plasma levels of 17-OH-CS on different days in the same subjects are noteworthy. It should be emphasized, too, that there was no obvious correlation between the severity of clinical manifestations and mean level of plasma 17-OH-CS.

Lack of variability in plasma 17-OH-CS is certainly an unspecific finding. Eik-Nes and Clark (11) noted the lack of diurnal variation in 4 patients following severe head injury, and Perkoff *et al.* (9) found significant loss of diurnal rhythm in clinical conditions associated with alterations in consciousness or sleep pattern. Moreover, in the patients of Eik-Nes and Clark the levels of plasma steroid were abnormally high. In patients with suprasellar extension of pituitary tumors Hökfelt and Luft (12) noted the absence of the normal variation in free plasma cortisol, emphasizing the role of hypothalamic control of ACTH secretion. The present authors have measured plasma 17-OH-CS in a limited number of cases of obesity, hypertension or osteoporosis not associated with Cushing's syndrome, and have found a normal variation of cortisol concentration.

Apparently Cushing's syndrome can be caused not only by cortisol-secreting adrenal tumors but by ACTH-secreting pituitary tumors (13–14). In most cases, however, the mechanism for the adrenal hyperfunction is not known. At present it seems probable that normal variations in plasma 17-OH-CS concentration are caused by changes in the rate of secretion of ACTH (9). The absence of diurnal variation in Cushing's disease is obviously not due to an inability of the adrenal cortices to respond further to increased stimulation by ACTH, as many cases of Cushing's disease exhibit a hyper-reactive response to ACTH (4, 15, 16). The results of the administration of an 11β-hydroxylase inhibitor in a few cases (17–19) indicate that the constant level of plasma cortisol probably cannot be explained on the basis of a lack of capacity of the pituitary to increase the secretion of ACTH. It is therefore not unreasonable to suggest that non-neoplastic Cushing's disease is in many cases caused by a disturbance in the function of an ACTH-controlling hypothalamic center. Such a view is

not in conflict with the known difficulty of inhibiting the adrenal cortex with glucocorticoids in patients with Cushing's disease due to adrenal hyperplasia (20).

The diagnosis of Cushing's syndrome can often be made from a typical clinical picture. Confirmation from laboratory studies, however, is always desirable. Moreover, an increasing number of patients are presenting in whom steroid studies are indispensable for a diagnosis. The existence of an abnormally increased secretion of cortisol in all cases of Cushing's syndrome is a reasonable assumption. In the absence of its direct measurement, the amount of cortisol secreted has to be estimated from the urinary excretion of cortisol and its metabolites. For routine purposes such metabolites are measured unspecifically as 17-KGS according to the method of Appleby *et al.* (6), or as Porter-Silber chromogens.

Recently, Cope and Black (21) emphasized the generally poor correlation between the urinary excretion of 17-KGS and Porter-Silber-reacting steroids on the one hand and the production of cortisol as measured by an isotope dilution method on the other hand. From their studies it appeared that, when steroid excretion was at high levels, the urinary 17-KGS output showed good correlation with the actual production of cortisol. In the normal range, however, the excretion of 17-KGS was poorly correlated with the secretion of cortisol. This fact might explain the findings of "normal excretion" of 17-KGS on repeated examination in Cushing's syndrome. In this connection, Cases 19, 20 and 21 are of interest; on repeated examinations there was no increase in the urinary excretion of 17-KGS.

In Patient G.S. (Case 19) the history extended back more than five years, at which time she had presented with severe hypertension, decreased renal function, amenorrhea and severe mental depression. Examination disclosed a rounded, plethoric face, atrophy of the skin with rubor, purple abdominal striae, easy bruisability, some hypertrichosis, and marked muscular atrophy. Abnormal laboratory findings included constant eosinopenia and lymphopenia and decreased glucose tolerance. During one month of preoperative studies, no increase was observed in the urinary excretion of 17-KGS or Porter-Silber chromogens. The patient was hyperreactive to intravenous ACTH. Results of the triamcinolone inhibition test (Table 3), however, were not clearly abnormal. At operation the adrenals appeared morphologically normal but were probably somewhat hyperplastic (7 and 8 Gm. respectively). Postoperatively there was regression of clinical symptoms with a return to normal habitus, as well as a marked decrease in blood pressure and improvement in mental status.

Patient A.D. (Case 20), a 51-year-old woman, was known to have had diabetes for three years. In addition she complained of increasing obesity and mental depression. On examination the obesity was found to be of

general distribution, the face was pale and there were no cutaneous changes or other characteristics of Cushing's syndrome. Moreover she excreted normal amounts of 17-KGS as well as of Porter-Silber chromogens. The abnormal findings included complete failure of triamcinolone inhibition (Table 3). A bone biopsy was interpreted as showing osteoporosis. The abnormal findings further included hyper-reaction to ACTH, as judged from the increase in plasma cortisol concentration and urinary excretion of 17-KGS. At operation, hyperplastic adrenals were removed (8 and 11 Gm. respectively).

Patient I.M. (Case 21), a 41-year-old woman, was seen by one of the authors for eight years; Cushing's syndrome was suspected on clinical grounds, but only recently was a chestnut-sized adrenocortical adenoma removed. In this case it was never possible to demonstrate an abnormally high urinary excretion of 17-KGS or Porter-Silber chromogens. However the plasma levels of the 17-OH-CS were extremely constant upon repeated examinations (Table 2). In addition it proved to be impossible to inhibit the urinary excretion of steroids with triamcinolone in a dosage of 8 mg. every six hours (Table 3). This patient complained of some mental depression, difficulty in weight reduction and repeated recurrences of duodenal ulcer. Clinical findings were sparse and included a rounded, somewhat highly colored face and labile hypertension. Moreover, her habitus had not changed and no other symptoms had developed during the eight-year observation period.

In Patient A.O. (Case 18) there was also a fairly normal excretion of 17-KGS. She sought medical advice in 1957 for a change in habitus, depression, amenorrhea and hypertension. She displayed the fully developed syndrome but refused operation and was thereafter treated by pituitary irradiation which was followed by an incomplete remission. At present she still has hypertension, amenorrhea and a habitus suggestive of Cushing's syndrome. Table 3 shows suggestive evidence for the presence of a continuing pituitary-adrenal disorder. However, failure of pituitary inhibition during administration of triamcinolone does not prove that she is actually secreting an abnormal amount of cortisol. Apropos, is the finding of a fairly constant level of cortisol in Patient L.L. (Case 22), who was in a state of complete clinical remission after partial adrenalectomy in 1955; this indicates that the pituitary-adrenal disorder was still present.

Cushing's syndrome does not always present in its well known, easily recognizable form but sometimes in an atypical form, lacking many of the classic characteristics. The making of a diagnosis in such cases can be challenging, as these patients may well present with a normal urinary excretion of cortisol metabolites. Because of the lack of normal variation in plasma 17-OH-CS concentration in most of the patients discussed here, it

seems reasonable to suggest that serial measurements of plasma cortisol will be an aid in the diagnosis of Cushing's syndrome.

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DIURNAL PATTERN OF PLASMA 17-HYDROXY-CORTICOSTEROIDS IN PRETECTAL AND TEMPORAL LOBE DISEASE

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ABSTRACT

Diurnal patterns of plasma 17-hydroxycorticosteroids were determined in 2 patients with localized pretectal area disease and in 2 with localized temporal lobe disease. All 4 patients exhibited abnormal patterns in contrast to 3 patients with disease localized elsewhere in the central nervous system, all of whom showed normal diurnal patterns. This provides further evidence that the temporal and pretectal areas of the brain are important in the regulation of pituitary release of corticotropin, and of the diurnal pattern of plasma 17-hydroxycorticosteroids, which is a reflection of such release.

A DIURNAL pattern of plasma 17-hydroxycorticosteroids (17-OH-CS) concentration has been well established (1, 2). Alterations of this pattern have been noted in patients with long-standing diffuse central nervous system disease accompanied by impairment of consciousness (3, 4), delirium or restlessness (4) and in patients with suprasellar tumor (5). In only one of the latter cases, however, was there no intrasellar involvement.

It has been suggested (4) that the diurnal variation in plasma 17-OH-CS concentration is dependent upon changes in the rate of secretion of corticotropin (ACTH) in response to a centrally located controlling mechanism. Evidence for central nervous system control of pituitary ACTH secretion has been steadily accumulating (6). The following pathway has been proposed (7): the reticular-activating system, lateral hypothalamus, septum, hippocampal formation, amygdala, hypothalamus, pituitary. Both amygdaloid (8) and hypothalamic (9) stimulation have been reported to cause elevation of the plasma 17-OH-CS level in the monkey, and bilateral fornix section in this species (10) is said to cause disruption of the diurnal variation in urinary 17-OH-CS. Lesions of the diencephalon and midbrain in the cat (11) cause a marked reduction in the adrenal secretion of aldosterone and hydrocortisone.

In view of these findings, the diurnal pattern of plasma 17-OH-CS was

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studied in 4 patients with focal central nervous system disease not involving the sella, and unaccompanied by a disturbance of consciousness.

METHODS AND MATERIAL

Plasma 17-hydroxycorticoids were measured by a modification of the Porter-Silber method (12). Two of the patients (R.S. and J.H.) had small lesions in the pretectal area characterized by paresis of upward gaze and convergent nystagmus upon upward gaze; the discreteness of these lesions was demonstrated by pneumoencephalography and myeloencephalography. The 2 other patients (F.N. and S.S.) had lesions in the left temporal lobe, characterized by psychomotor seizures reflected in a focally abnormal electroencephalogram.

The salient clinical data are presented in Table 1. The history, physical findings and laboratory studies of gonadal, thyroidal and adrenal activity failed to reveal any evidence of intrinsic endocrine dysfunction.

Controls for this study consisted of 3 patients with circumscribed central nervous system disease (sphenoid ridge meningioma, left occipital lobe cyst, and cerebellar tumor, respectively).

RESULTS

The pattern of diurnal variation of 17-hydroxycorticosteroids observed in normal subjects by others (1, 2) and by ourselves in 6 cases, consists of a peak (20 to 40 μg. per 100 ml.) occurring between 4 a.m. and 8 a.m., and then a progressive fall during the remainder of the day.

Figure 1 depicts the diurnal variations of plasma 17-OH-CS in our 4 patients. Repetition of the determinations in 2 of these patients showed a similar diurnal pattern. Further studies will have to be performed to see whether this reproducibility is maintained. All of the curves in Figure 1 show a marked deviation from the normal pattern, with peak levels considerably in excess of any observed under normal circumstances in our laboratory. Similar findings have been reported (3) in patients with severe brain damage.

All 3 control patients showed essentially normal diurnal patterns of plasma 17-hydroxycorticosteroids.[1]

These data indicate that disruption of the diurnal pattern of plasma 17-OH-CS occurs in patients with focal disease in the region of the pretectal area and temporal lobe, unaccompanied by disturbances of consciousness, increased intracranial pressure or evidence of pituitary dysfunction. This is further evidence, in conjunction with the previously cited work of Green (7) and Mason (8, 10), that these areas are important in the regulation of

[1] The actual diurnal variations of plasma 17-OH-CS in these 3 patients were: (*a*) Sphenoid ridge meningioma—4 a.m., 7.1 μg./100 ml.; 8 a.m., 19.3 μg.; noon, 15.4 μg.; 4 p.m., 10.1 μg.; 10 p.m., 2.8 μg. (*b*) Left occipital lobe cyst—4 a.m., 19.0 μg./100 ml.; 8 a.m., 18.0 μg.; noon, 18.7 μg.; 4 p.m., 8.2 μg.; 10 p.m., 3.2 μg. (*c*) Cerebellar tumor—4 a.m., 16.0 μg./100 ml.; 8 a.m., 18.0 μg.; noon, 9.0 μg.; 4 p.m., 7.0 μg.; 10 p.m., 4.0 μg.

TABLE 1

Patient Initials, age & sex	Diagnosis	Neurologic findings	State of consciousness	Sella	Pneumoencephalogram	Electroencephalogram
J.H., 34, F	Pretectal mass, presumably metastatic (breast)	Ataxia, headache, blurring of vision; paresis of upward gaze; weakness of left upper extremity	Intact	Normal	Mass just to rt. of aqueduct, inf. to III vent.	Normal
R.S., 45, M	Pretectal mass; ?neoplasm or hemorrhage	Attacks of weakness of knees; paresis of upward gaze; retractory nystagmus; left hemiparesis	Intact	Normal	Normal IV vent. and aqueduct; no air in III vent.	Abnormal fronto-parietal activity accentuated on right
F.N., 53, F	Left temporal lobe disease; etiology unknown	Psychomotor seizures for 2 yrs.; no abnormal signs	Intact	Normal	Normal	Minimal abnormal diffuse bursts, 6.5–8 cps, left temporal region
S.S., 40, F	Left temporal lobe disease; etiology unknown	Psychomotor seizures for 5 yrs.; no abnormal signs	Intact	Normal	Normal	Abnormal diffuse bursts, left temporal region

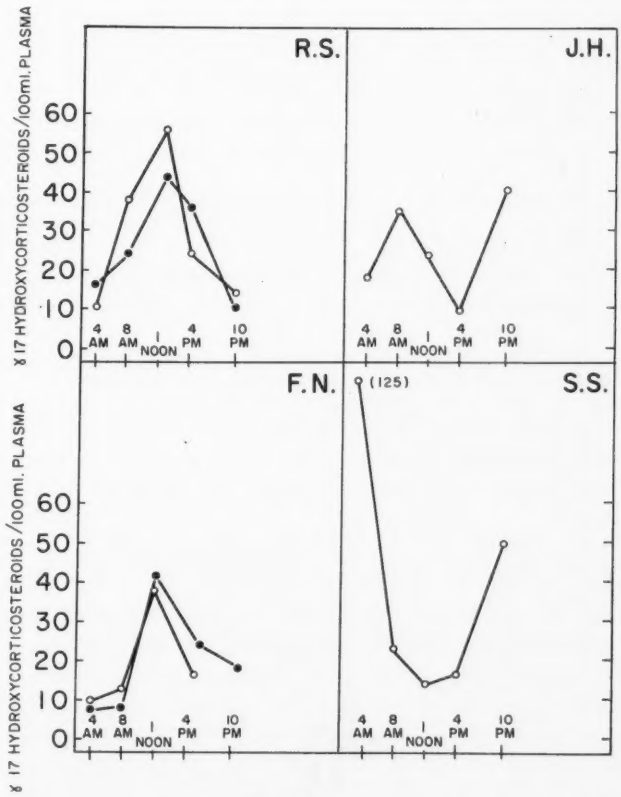

FIG. 1. Diurnal pattern of plasma 17-hydroxycorticosteroids in patients with temporal lobe and pretectal disease. (Duplicate determinations on Patients R.S. and F.N. were performed at intervals of one week.)

pituitary release of ACTH and the diurnal pattern of plasma 17-hydroxycorticosteroids, which is a reflection of such release.

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NUCLEAR CHANGES IN THYROIDAL EPITHELIUM FOLLOWING RADIATION FROM RADIOIODINE*†

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ABSTRACT

A quantitative histochemical method was used to measure desoxyribonucleic acid (DNA) in the nuclei of rat thyroid cells after the animals had been subjected to varying doses of radioiodine (I^{131}). Only nuclei with diploid values for DNA were found in the normal thyroid. The administration of thiouracil to nonradiated rats produced nuclei containing increased amounts of DNA, up to twice the diploid value—the level at which cell division normally occurs. After moderate doses of radiation, bizarre nuclear forms containing unusually large quantities of DNA were found. These forms were also observed after very small doses of radiation followed by administration of thiouracil. Thus, seemingly insignificant doses of radiation, which did not impair physiologic function, produced latent changes which became evident only with an added stimulus for cell division. To complement these observations, it was found that even small amounts of radiation impaired the ability of the gland to hypertrophy when a goitrogen was given. The increase in DNA indicates that the cells were preparing to divide, either under the influence of an endogenous stimulus, or in response to thiouracil. DNA continued to accumulate in abnormal quantities in these nuclei after cell division should have occurred. It is concluded that that radiation in some way alters the genetic mechanisms, perhaps by destroying chromosomes, so that the abortive cell division results in amounts of DNA which do not fall into recognizable nuclear classes. The clinical significance of these changes in the thyroid is speculative.

IN PREVIOUS investigations (1, 2) in man and rats it was observed that, following damaging doses of radioiodine (I^{131}), there appeared thyroidal epithelia containing some bizarre nuclear forms and what seemed to be unusual concentrations of chromatin material. This was confirmed by other investigators in animals (3–9) and in patients treated with I^{131} (10–13). The nuclear changes in the patients (2) not only appeared in the early post-radiation period, but were found as long as eight years

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later. Among rats given varying amounts of I^{131}, it was observed that small doses of I^{131} might not result in the strange nuclear forms until the thyroids were subsequently stimulated by thiouracil (1). The impression was gained that following radiation a stimulus was necessary to produce the bizarre nuclear forms, whether that stimulus resulted from physiologic impairment of the function of the thyroid tissue (*e.g.*, following large doses of radiation) or from the administration of an antithyroid compound (*e.g.*, a goitrogenic drug given after small doses of radiation).

Upon examination of hematoxylin and eosin preparations (1, 2) the assumption was made that some of the cells in the radiated thyroids contained considerably more chromatin material than others. However, it is generally acknowledged (14) that essentially all somatic cells in animals of a given species contain the same amount of chromatin, since they contain the same number of pairs of chromosomes. The nuclei of somatic cells are classified as diploid; they contain twice the amount of chromatin in the nuclei of sperm, which are called haploid. It is also acknowledged (14, 15) that the chromatin of the diploid nucleus increases by a factor of 2 preceding cell division and that the division results in two cells, each bearing the original amount of chromatin. The proliferation of malignant cells is usually faster than that of normal tissue, and the amounts of chromatin not only are variable but are sometimes greater than twice the diploid value (14, 16, 17). Although the apparent increase in chromatin material manifested by bizarre nuclei in the previous studies of radiated thyroids (1, 2) suggested abnormal cellular proliferation, there was also evidence that some of these radiated cells failed to divide. This was manifested by impairment to the development of the glandular hypertrophy usually produced by the administration of thiouracil; indeed, some glands containing these bizarre nuclei failed to enlarge at all.

Boivin, Vendrely and Vendrely (18) using chemical methods, Swift (19) using cytochemical methods, and Leuchtenberger, Vendrely and Vendrely (20) using both methods, demonstrated that most somatic cells of animals of the same species contain approximately the same amount of desoxyribonucleic acid (DNA) and that sperm contain only half this amount. Since DNA represents the major component of chromatin in the nucleus, the measurement of this substance in nuclei of the epithelia of previously radiated thyroids was planned. Thus, the object of these experiments has been to measure and compare the DNA content of thyroidal nuclei, not only within the same gland, but in glands of different animals in which the dose of radiation, the survival time following radiation, and the presence of a stimulus such as administration of thiouracil were varied experimentally.

METHODS

Experimental design

In general, the experiments consisted of giving rats various doses of I^{131} and later measuring the amount of DNA in individual nuclei of the thyroid after different periods of survival. In some instances a stimulus to thyroid growth was applied following radiation.

Two hundred and seven immature male white rats of the Sprague-Dawley strain and from the same source as the animals used in the previous experiments (1) were selected for this study. Each weighed between 110 and 135 Gm.

A standard diet (Purina Fox Chow, which contains an abundance of iodide) was given for ten days following arrival of the rats from the supplier. In order to insure a satisfactory uptake of I^{131} by the thyroid, the animals were then given a modified Remington diet (1), containing 0.025 microgram of iodide per gram of food. These animals, suffering from iodine deficiency and the resulting hyperplasia of the thyroid, were divided into appropriate groups and given varying intraperitoneal doses of I^{131}. Sodium iodide, 0.013 microgram per dose, was used as carrier. Following the period of iodine deficient diet and the administration of I^{131}, the rats were given a balanced diet (devised by Gassner) known to contain 0.4 to 0.7 microgram of iodine per gram of food. This was shown by Gassner (21) to be a diet supplying an adequate but not excessive amount of iodine for rats. The animals were maintained with this diet for the duration of the experiments. A few rats, serving as absolute controls,[1] were given no Remington diet or I^{131} but were given the Gassner diet throughout the study. During the period of survival all animals were weighed at intervals.

Two series of animals will be described. *The first series* was given 0, 10, 30, or 100 μc. of I^{131} after receiving the Remington diet for nineteen days. This diet was continued for three additional days, and thereafter the Gassner diet was used. The uptake of I^{131} and the degree of thyroid hyperplasia induced by the iodine deficient diet were determined in a few animals sacrificed forty-eight hours after I^{131} had been administered. Nine weeks later the groups of animals were subdivided so that some might be given thiouracil (0.1 per cent in the drinking water) until the termination of the experiment. Some of each group were sacrificed at sixteen weeks, and the remainder at twenty-six weeks after the administration of I^{131}.

The second series of experiments was for the purpose of re-exploring more thoroughly the radiation effect of lower doses of I^{131} for longer periods of time, with and without the added stimulation of thiouracil. Some modifications were made in the experimental design. The preliminary period of Remington diet was fourteen days and the Gassner diet was introduced the day following administration of I^{131}. The rats of this series were given 0, 5, 10, 20, or 40 μc. of I^{131}. None was sacrificed soon after receiving I^{131}, but *in vivo* measurements of I^{131} uptake were made at various times between forty-eight hours and seven days. In contrast to the first series of experiments, the administration of thiouracil in the second series was started on the fourteenth day after the dose of I^{131}. No animals were sacrificed until the twenty-seventh week following radiation; the remainder were sacrificed in the eighty-second week.

In both series of experiments the animals were killed with ether; the thyroids were immediately and rapidly removed, weighed, and placed in fixative. The weight of the

[1] In contradistinction to other control animals which received the Remington diet but did not receive I^{131}.

body and the weight of the anterior pituitary (only in the second series) were also determined. Each time experimental animals were sacrificed, some animals which had received no I^{131} or thiouracil were also killed.

Instrumentation and preparation of tissues

A method of microspectrophotometric analysis designed by Pollister and associates (19, 22–24) was used. The method utilizes the quantitative Feulgen staining reaction of aldehydes with fuchsin sulfurous acid, the aldehydes being liberated by acid hydrolysis which breaks the purine-sugar linkage in the DNA molecule. Under the conditions of the experimental method, the Feulgen reaction is specific for desoxyribonucleic acid (25). The method of preparing the tissue for study was that of Pollister and associates.[2]

Although Carnoy's acetic alcohol (1:3) is an unsuitable fixative for precise morphologic studies with conventional stains, it was used in this study because it precipitates the nucleoproteins most completely and at the same time removes lipids and other chemical constitutents not pertinent to the study of DNA. After fixation overnight, the blocks of tissue (measuring no more than 3 mm. in thickness) were dehydrated and placed in paraffin. Because animals were sacrificed at different times, and because it was necessary to stain all tissues simultaneously, the paraffin blocks were stored until all the tissues were available. After the sections were cut[3] and stepwise returned to water, hydrolysis of DNA was accomplished by exposure to 1 *N* hydrochloric acid at 60° C. for exactly twelve minutes. The sections were washed, placed in Schiff's solution for two hours, washed, and placed in bleaching solution (20 ml. of 1 *N* hydrochloric acid, 20 ml. of 10 per cent aqueous sodium metabisulfite, 360 ml. of water) for three successive applications of ten minutes each. After they were thoroughly washed and dehydrated, they were mounted in synthetic resin. Great care was taken to time each step of this procedure precisely.

The intensity of the Feulgen color reaction, upon which the measure of DNA depends, varies as a result of a number of conditions. For instance, one of the most important steps in the preparation of the material is the hydrolysis. The extent of hydrolysis must be just sufficient to produce the maximal intensity of staining. If hydrolysis is carried beyond this point, decreased intensity of staining results. Owing to this factor and a number of other variables in the staining reaction it is clear that *the nature of the reaction is such that absolute quantititative measurements are not possible. However, a measure of the relative amounts of DNA in the nuclei is feasible* when the tissues are fixed immediately and are simultaneously carried through the steps of preparation, including the mounting and staining of sections for comparison side by side on the same slide.

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[2] The authors are greatly indebted to Dr. Cecilie Leuchtenberger, formerly of this University, and to Dr. Arthur W. Pollister of Columbia University, for their many helpful suggestions in setting up this method of study in our laboratory. We wish particularly to thank Dr. Leuchtenberger for assistance with some of the staining of the preparations in the early phase of this work. It has served as a check on the methodology used in our laboratory.

[3] Exploratory studies were made during the first full-scale experiment to find the optimal thickness for slices of thyroid tissue. Thin sections were desirable, but it was necessary that they be of sufficient thickness to permit a satisfactory yield of whole nuclei for measurement. Accordingly, some sections were cut at 6, 12, and 18 microns. Appraisal of the frequency of whole nuclei prompted the adoption of 8 microns as the appropriate thickness for the study of these thyroid tissues.

The measurement of DNA (19, 22–24) in individual cells was carried out microscopically under high magnification, and was based on the measurement of absorption of monochromatic light by the Feulgen stain. This is an application of the Beer-Lambert law, *i.e.*, the amount of light energy absorbed is a function of the number of dye molecules bound to the substances in the path of light. The number of molecules varies with the concentration or the thickness of the absorbing layer. Although these two values may vary, the specific absorption of the substance is a constant; thus loss of light is the product of concentration, thickness, and the constant. Since the colored product of the staining procedure is most accurately measured at 546 mμ, monochromatic light at this wave length is used. Parallel light passes through the section of tissue into the microscope. The instrument is so arranged that a direct-vision magnifying ocular or a photometer may be swung into position over the image. The image of the nucleus may be observed and its diameter measured with a micrometer. When a nucleus is perfectly centered in the field, a diaphragm aperture is interposed between the microscope and the photometer. An aperture size (constant diameter for these experiments) which represents an area somewhat less than the area of the nucleus is used. The photometer is so adjusted that a full-scale deflection on the galvanometer occurs when the same size of aperture is used over a blank area of the slide. This blank reading represents 100 per cent transmission. A similar reading of intensity is made when the same aperture is centered perfectly over a given nucleus. The intensity is thus measured for a cylindrical plug of nucleus. *Transmission* is calculated as: $T = I_n/I_o$, where I_n is the intensity of the light passing through the plug of nucleus and I_o is the intensity of the light passing through the blank area. The transmission does not vary directly with the number of absorbing molecules but decreases logarithmically with a linear increase in the number of absorbing molecules. *Extinction* (E) is defined as varying directly with the number of absorbing molecules. Thus, $E = \text{Log } (1/T)$ or $\text{Log } I_o/I_n$. Since the major and minor axes of an ellipsoid nucleus have been measured with an ocular micrometer, an average diameter of the nucleus can be determined. Because the plug represents a known percentage of the volume of the sphere, the extinction for the sphere can be calculated. Arbitrary units are expressed $E\ (C^2/F)$, where C = the radius of the plug measured, and F = the fraction of the total nuclear volume which is included in the cylinder.

Careful attention to a number of technical details was necessary to make this method of microspectrophotometry dependable. Proper alignment of the entire optical system was imperative. Linearity of the response of the phototube was considered not only from the standpoint of exact centering of the aperture on the cathode of the photomultiplier tube but from the standpoint of uniformity of the field of light and the constancy of the light source. All measurements were made in a dark room to avoid extraneous light, and all possible light scatter and internal reflections within the optical system were reduced to a minimum. All measurements of nuclei were made with an aperture (plug) large enough to give a true average transmission, thus reducing the error produced by clumping of chromatin. Only whole nuclei which were shown to be complete and free from superimposition were measured. By focusing up and down and observing the apparent progressive change in refraction rings, the observer could distinguish a complete nucleus from one which had been transected. The avoidance of incomplete nuclei eliminated one source of false low values for DNA. Calculation of the volume of an ellipsoidal or irregular nucleus may have been a source of error, and in this particular study some of the irregular shapes encountered obviously led to error. Since it was these particular nuclei which were of primary concern, they were measured as they were observed in the random sampling, and a reasonable average diameter was estimated for each nucleus.

Tissue sections from the thyroids of animals of different groups and subgroups were mounted together on slides in a variety of combinations. Although not all experimental conditions could be included, sections representing as many as 11 different animals were placed on a single slide. The presence of multiple sections on the same slide insured identical exposure in the different solutions with respect to concentration, temperature, and the intervals of time for each step in the preparation. For practical purposes the results were equally reliable when two slides were processed back to back through the various solutions.

Fifty nuclei were measured in each section of thyroid. The amount of DNA for each nucleus was calculated, and a standard deviation of the mean DNA per nucleus was determined for each thyroid.

Some duplicate sections from thyroids of animals in the second series of experiments were mounted and stained with hematoxylin and eosin for the purpose of evaluating the height of the follicular cells. Cell heights were appraised by measuring with an ocular micrometer the heights of 100 cells (an average cell from each of 100 follicles) when the tissue was observed under oil immersion magnification, according to the method of Starr and Rawson (26).

RESULTS

First series of experiments (Table 1)

Three sample animals from each of the four groups (0, 10, 30 and 100 μc.) that had received the Remington diet were sacrificed forty-eight hours after I^{131} had been given. In addition, 3 animals which had not received the Remington diet were sacrificed. This was done to determine the degree of glandular hypertrophy and the actual thyroidal uptake of I^{131}. Thyroid weights resulting from the initial iodine deficiency averaged 5.8 mg. per 100 Gm. of body weight (range, 6.0 to 13.0 mg.) as compared to 3.4 mg., per 100 Gm. of body weight in the animals which had received no Remington diet.

The thyroidal 48-hour uptake of I^{131} in all groups that had received the Remington diet was 11.8 to 47.6 per cent of the dose, as determined by measurements made on the thyroids at the time of their removal. Unfortunately, serial determinations based on *in vivo* counts from which to calculate the observed half-life of I^{131} in the gland were not available. However, on the basis of previous observations (1) obtained under similar conditions, it was assumed that the observed half-life of I^{131} in these animals was about 2.5 days. From the range of I^{131} uptake and the average weight of the gland, the radiation delivered to the thyroid was estimated.[4]

The amount[5] of I^{131} in the thyroids of representative animals of the first

[4] There was considerable variability in the I^{131} uptake and in the size of the thyroids. Because animals were caged in groups when receiving the iodine-deficient diet, any animal which did not survive served as a source of iodine for one or two of his cage mates. His thyroid was promptly removed and eaten by a cage mate, presumably making the recipient less iodine deficient than the others.

[5] The amount (μc./mg. thyroid tissue) was based on the corrected uptake (half-life, 2.5 days) to twenty-four hours after I^{131}.

TABLE 1. *First Series.* CHANGES IN THYROID WEIGHT, NUCLEAR VOLUME, AND DNA WITHIN THE NUCLEI OF THYROIDAL EPITHELIUM AFTER 0, 10, 30 OR 100 μc. OF I^{131}, WITH AND WITHOUT THE STIMULATING EFFECT OF THIOURACIL

Animal Nos.	Dose of I^{131} (μc.)	Thiouracil	Avge. weight of thyroid (mg./100 Gm. body wt.)	Avge. mean nuclear vol (μ^3)	Avge. of mean DNA* among animals of group	Avge. std. dev.** DNA within each animal
			16 Weeks			
11–13†	0	No	5.6	47	1.19 ± .078	.029
14–16	0	Yes	13.7	75	1.40 ± .120	.052
17–19	10	No	4.0	52	1.20 ± .102	.043
20–22	10	Yes	4.5	81	1.68 ± .161	.085
23–25	30	No	2.7	72	1.47 ± .145	.085
26–28	30	Yes	4.7	90	1.89 ± .211	.121
29–31	100	No	1.6	Extensive distortion		
32–34	100	Yes	1.7	Extensive distortion		
			26 Weeks			
72–75‡	0	No	5.0	57	1.51 ± .069	.035
70	0	No	5.5	66	1.57	.045
71	0	Yes	11.0	74	1.53	.057
47–51	10	No	3.6	74	1.59 ± .116	.068
52–56	10	Yes	5.1	82	1.72 ± .095	.085
57–58	30	No	3.4	81	1.58 ± .085	.075
59–63	30	Yes	5.1	84	1.84 ± .261	.084
65	100	No	2.7	78	2.21	.102
66–69	100	Yes	2.1	61.5	1.87 ± .057	.106

* A mean DNA in arbitrary units per nucleus for each animal was determined, and from these the average and standard deviation was calculated to illustrate similarity within the group of animals. The number following the ± sign is the standard deviation.

** The average of the spread in the amounts of DNA per nucleus in each of the animals of the group (square root of average of squared standard deviation).

† Controls: Remington diet only.

‡ Absolute controls: No Remington diet; no radiation.

series at the three dose levels, and the approximate amount of radiation delivered, were as follows:

Total dose of I^{131}	Amt. in thyroid (μc./mg.)	Radiation delivered to thyroid (rads)
100 μc.	1.6 to 4.2	80,000 to 220,000
30 μc.	0.44 to 0.92	23,000 to 46,000
10 μc.	0.07 to 0.09	3,500 to 4,800

As shown in Figure 1, the administration of thiouracil to non-radiated animals caused not only an increase in the amount of DNA but also a pro-

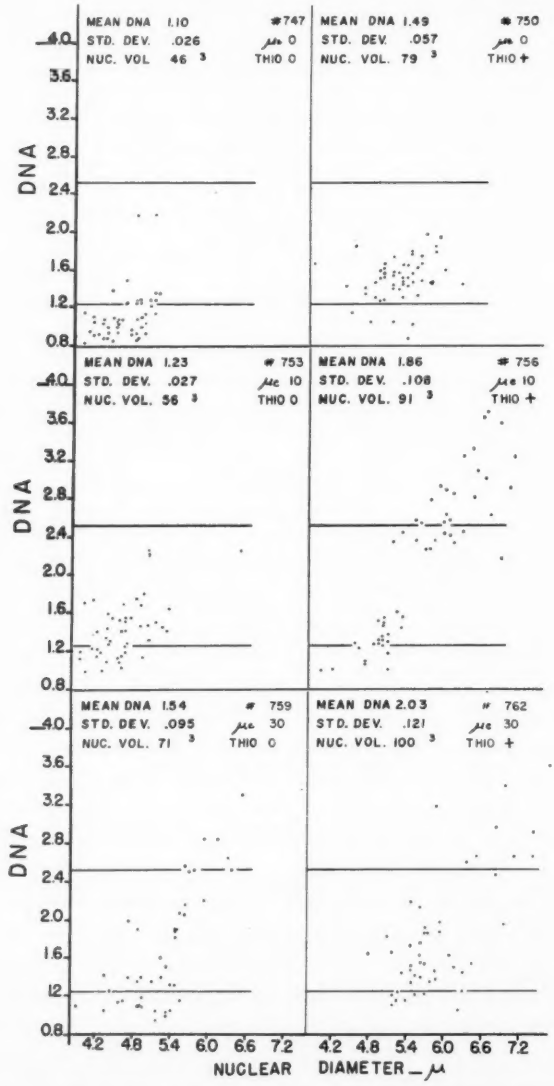

FIG. 1. Scatter graph representing the amount of DNA and the volume of individual nuclei of thyroid epithelium 16 weeks after 0, 10 or 30 μc. of I^{131}, with and without subsequent stimulation by thiouracil.

The data from a representative animal of each group are shown. Each point on the graph represents a single cell. Nuclear volume is expressed in cubic microns. DNA is expressed in arbitrary units. The horizontal line at 1.2 units of DNA in each graph is the diploid value and represents the mean DNA value as determined from thyroids of

portional increase in the volume of the nuclei. This result was expected because cell division had been stimulated and hypertrophy of the gland had occurred (Table 1). The increase in DNA resulted in considerable spread among the values for the amount of DNA in the nuclei, but this increase rarely exceeded twice the diploid value.

No increase in DNA occurred within sixteen weeks after the administration of 10 μc. of I^{131} alone. However, when thiouracil was given during the latter part of the 16-week interval, there was an increase of DNA in individual nuclei (Fig. 1); indeed, the amount of DNA increased to more than twice the diploid value in some nuclei. Increases up to twice the diploid value might normally be expected as a result of a stimulus to cell division, but greater increases suggest an abnormality in the process of cell division. When the 10-μc. animals were permitted to survive twenty-six weeks rather than sixteen, the amount of DNA increased to more than twice the diploid value, even when the stimulus of thiouracil had not been applied. This change, however, was not as great as that produced by thiouracil at sixteen weeks. When the effect of thiouracil was superimposed at this later date, the aberration in DNA appeared the same as at sixteen weeks (Table 1).

The 30-μc. dose of I^{131} resulted in an increase in DNA within sixteen weeks without the stimulus of thiouracil (Fig. 1 and Table 1). When thiouracil was given, both the mean increase and the variation in the amount of DNA among the nuclei of each gland were more pronounced than when the animals had received only 10 μc. of I^{131} (see the last column in Table 1). The passage of time (to twenty-six weeks) seemed to add relatively little to these changes. It is noteworthy that, although some cells contained abnormally large amounts of DNA, indicating abnormal or

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animals which had received no I^{131} or thiouracil. The second horizontal line at 2.4 units represents twice the diploid value and is the theoretical point at which these cells would be expected to divide. From above downward, the graphs represent animals which received increasing amounts of radiation. The graphs on the right represent animals that received thiouracil; those on the left represent animals that received no thiouracil. No nucleus contained DNA in amounts greater than twice the diploid value in the normal untreated animals (upper left). The stimulus produced by thiouracil (upper right) resulted in an increase in DNA and nuclear volume, but values greater than twice the diploid value were rarely seen in this group of animals. The effects of 10-μc. and 30-μc. doses of I^{131} are seen in the middle and lower graphs, respectively. When I^{131} was given in small amounts and the stimulus of thiouracil was superimposed, variations in the amount of DNA in the nuclei became evident. Many nuclei were found that contained amounts of DNA greater than twice the diploid value.

thwarted cell division, the administration of thiouracil still produced a significant increase in thyroid weight.

The observations on animals receiving 100 μc. of I^{131} were not suitable for comparison with those on other groups because of the extensive alteration in the thyroids and the relatively poor survival of the animals. There was considerable variation in the degree of destruction of the thyroids in this group, even though the animals had been given identical doses of I^{131}. This variation was probably based on differences in the uptake of I^{131} by the thyroid or in sensitivity of the gland to the radiation.

Growth, as judged by body weight, was followed in these young animals. Although thiouracil impaired body growth, the radiation damage produced by I^{131} in doses less than 100 μc. did not affect the growth curve significantly. It is probable that any subtle thyroid hormone deficiency resulting from lesser doses of radiation might not have become significant until the animals had become almost fully mature. Thus, the effect of a deficiency in thyroid hormone on body growth would not have been evident.

The nuclei of the thyroids of the control animals which had sustained a temporary period of iodine deficiency and were then permitted to survive for sixteen weeks, had a lower DNA content than those of the absolute controls. This decrease in DNA content was present even when the previously iodine-deficient glands had returned to normal size.

Second series of experiments (Table 2)

In this series, the effect of a temporary iodine-deficient diet was given further consideration. In the control animals which had suffered only temporary iodine deficiency, the thyroid nuclei were again found to have lower DNA content than in the absolute controls (Table 2). In these experiments, twenty-seven weeks had elapsed following the two-week period of iodine-deficient diet. Comparisons could not be made after eighty-two weeks, for there were no surviving animals at that time among those that had sustained only iodine deficiency.

The thyroidal uptake of I^{131} in this series was determined on the basis of *in vivo* counts at various intervals from seventy-two hours to seven days following the dose of I^{131}. The average uptake was 16 per cent in seventy-two hours. The highest recorded uptake by this *in vivo* method was 27.1 per cent and the lowest was 11.3 per cent. On the basis of an estimated half-life of 2.5 days, the thyroidal uptake of I^{131} was judged to be approximately the same as that in the first series of animals.

Thiouracil caused some increase in nuclear DNA content in non-radiated animals; the increase within individual cells, however, was not greater than would be expected for normal cell division, *i.e.*, up to twice the diploid value (Table 2, animals #136–139 and #132–135; Fig. 2, representative

TABLE 2. *Second Series.* CHANGES IN THYROID WEIGHT, NUCLEAR VOLUME, AND DNA WITHIN THE NUCLEI OF THYROIDAL EPITHELIUM AFTER 0, 5, 10, 20 OR 40 μc. OF I^{131}, WITH AND WITHOUT THE STIMULATING EFFECT OF THIOURACIL

Animal Nos.	Dose of I^{131} (μc.)	Thiouracil	Avge. wt. of thyroid (mg./100 Gm. body wt.)	Avge. mean nuclear vol. (μ^3)	Avge. of mean DNA* among animals of group	Avge. std. dev.** DNA within each animal	Avge. ratio Pituitary (mg.) / Body wt. (100 Gm.)
			27 Weeks				
172–175† Absolute controls	0	No	5.5	68	1.33 ± .041	.035	2.9
136–139‡ Controls	0	No	5.2	64	1.17 ± .084	.029	3.0
132–135	0	Yes	12.7	75	1.36 ± .119	.056	4.0
144–147	5	No	3.9	62	1.38 ± .113	.056	3.3
140–143	5	Yes	8.1	72	1.44 ± .206	.081	3.8
152–155	10	No	4.0	70	1.35 ± .108	.054	3.3
148–151	10	Yes	7.5	92	1.65 ± .102	.093	4.5
160–163	20	No	3.6	69	1.40 ± .072	.063	3.5
156–159	20	Yes	7.7	105	1.76 ± .179	.0 6	5.2
168–171	40	No	2.7	76	1.52 ± .091	.075	3.0
164–167	40	Yes	5.3	92	1.67 ± .251	.076	4.5
			82 Weeks				
228–230† Absolute controls	0	No	7.1	68	1.37 ± .050	.029	2.7
—	—	—	—	—	—	—	—
210–214	0	Yes	18.5	68	1.43 ± .161	.055	4.3
216–219	5	No	5.5	64	1.25 ± .167	.063	3.9
215	5	Yes	65.2	66	1.53	.044	2.9
—	—	—	—	—	—	—	—
220	10	Yes	8.9	87	1.83	.088	3.9
—	—	—	—	—	—	—	—
221–222	20	Yes	5.7	91	1.78 ± .227	.103	5.7
226–227	40	No	4.0	96	1.64 ± .453	.081	3.8
223–225	40	Yes	4.3	90	1.73 ± .275	.088	5.0

* A mean DNA in arbitrary units per nucleus for each animal was determined, and from these the average and standard deviation was calculated to illustrate similarity within the group of animals. The number following the ± sign is the standard deviation.

** The average of the spread in the amounts of DNA per nucleus in each of the animals of the group (square root of average of squared standard deviation).

† Absolute controls: No Remington diet; no radiation.

‡ Control: Remington diet only.

animals #136 and #132.) The thyroids of these thiouracil-treated animals were markedly hypertrophied (Table 2).

In animals that had been given the temporary Remington diet followed by doses of 5, 10, 20 or 40 μc. of I^{131} and were permitted to survive twenty-seven weeks without thiouracil, the increases in mean DNA per nucleus were somewhat greater than in animals that had been given only the temporary Remington diet but no radiation. Although comparisons made between the values for the mean nuclear DNA of the several radiation groups were not particularly noteworthy, the variation in the amounts of DNA among the nuclei of individual thyroids was considerably increased,

as shown in the seventh column of Table 2. As in the first series of experiments, this variation among the nuclei was further increased when thiouracil was given. A dose of 5 μc. of I^{131} (amounting to approximately 0.08 rads) followed by the administration of thiouracil caused individual nu-

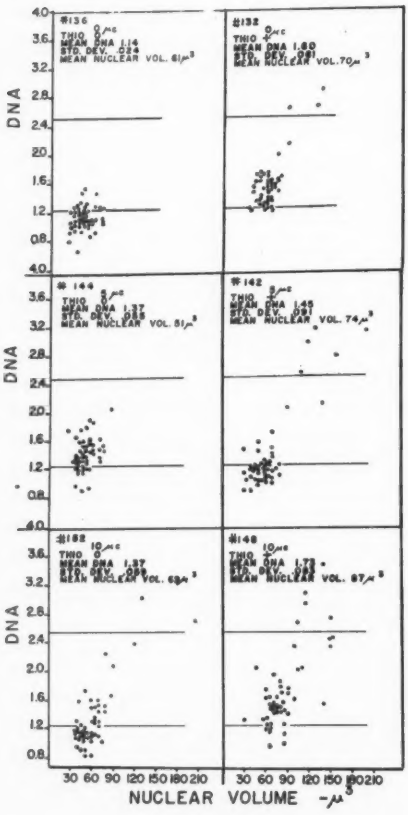

FIG. 2A

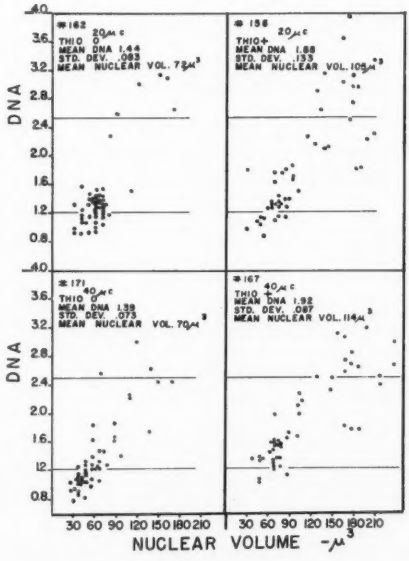

FIG. 2B

FIG. 2 (A and B). Scatter graphs representing the amount of DNA and the volume of individual nuclei of thyroid epithelium 27 weeks after 0, 5, 10, 20 or 40 μc. of I^{131} with and without the subsequent stimulation by thiouracil.

An increase in DNA was produced by the administration of thiouracil. A 5-μc. dose of I^{131} alone produced some increase above the diploid value of DNA in the nucleus, but when thiouracil was administered in addition, a few cells showed amounts of DNA greater than twice the diploid value. With 10, 20 or 40 μc. of I^{131}, occasional cells were found to contain DNA in amounts more than twice the diploid value. The addition of thiouracil at each dose level caused a further increase, not only in DNA content but in nuclear size.

clear changes that could be distinguished from those in animals that had received no radiation. These nuclear changes were represented better by individual cells than by the average of a population of cells in which many of the members were relatively normal; actually, a minority of the cells exhibited the changes concerning which there was most interest. There were micronuclei as well as macronuclei, as shown by the nuclear volumes. The variation in nuclear DNA content and in nuclear volume is therefore

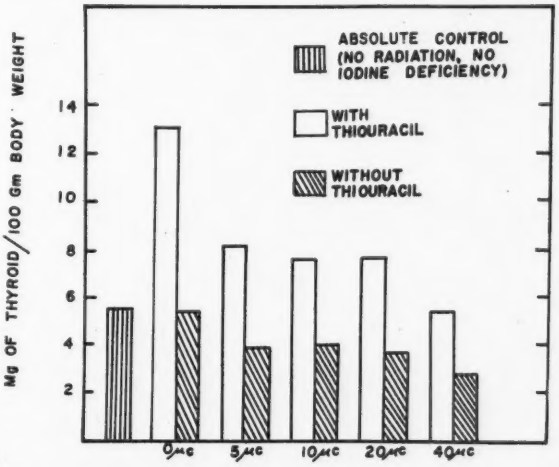

FIG. 3. Effect of small doses of I^{131} on thyroid weight and on the capacity for glandular hypertrophy in response to administration of thiouracil.

In spite of the impairment to glandular hypertrophy, many nuclei in these thyroids appeared to be preparing for mitosis.

best illustrated by a scatter graph, as in Figure 2. The effects of radiation and thiouracil on DNA, as observed in the first series of animals, were confirmed. In addition, these effects were shown to exist over longer periods.

It was also demonstrated that doses of I^{131} as small as 5 μc. could impair the ability of the gland to hypertrophy when thiouracil was given (Fig. 3). Whereas the non-radiated thyroids which weighed 5.2 mg. per 100 Gm. of body weight increased to 12.6 mg. per 100 Gm. when thiouracil was given, the thyroids of the 5-μc. radiated animals increased to only 8.1 mg. per 100 Gm. of body weight under the same stimulus.

The degree of hypertrophy of the thyroid produced by thiouracil following radiation differed in the first and second series of experiments (Table 3). In the first series, administration of thiouracil was not started until seven weeks had elapsed following the dose of radiation. The hypertrophy that could be produced after this delay was considerably less than in the second

series, in which thiouracil was started more promptly (fourteen days) after radiation. Even though the cells had received most of the radiation within fourteen days, the capacity for cell division was gradually lost over a longer period of time. This delayed loss of capacity for hypertrophy was originally demonstrated by Skanse (27) and more recently by Doniach and Logothetopoulos (7).

The prolonged administration of thiouracil again interfered with growth. The only animals in which growth was impaired after receiving I^{131} were those which had received 20 μc. It is of interest that this particular group

TABLE 3. THE INCREASE IN THYROID WEIGHT PRODUCED BY THE PROMPT OR DELAYED ADMINISTRATION OF THIOURACIL FOLLOWING A DOSE OF I^{131}

Dose of I^{131} (μc.)	Thyroid weight (mg./100 Gm. of body wt.)	
	Series I, delayed admin. of thiouracil*	Series II, prompt admin. of thiouracil†
0	13.7	12.7
5	—	8.1
10	4.5	7.5
20	—	7.7
30	4.7	—
40	—	5.3
100	1.7	—

* Thiouracil begun nine weeks after I^{131} and continued for seven weeks.
† Thiouracil begun two weeks after I^{131} and continued for twenty-five weeks.

also displayed the greatest change in nuclear DNA content and nuclear volume.

Since marked nuclear irregularities (Fig. 4) were produced by small doses of radiation plus the stimulus of thiouracil, or by larger doses of radiation alone, evidence was sought to prove our earlier hypothesis (1) that subtle thyroid deficiency might have caused an endogenous stimulus. Maloof (6) has shown that hypophysectomy or administration of thyroxine retards the development of these nuclear changes. Accordingly, the pituitaries were weighed in the second series of animals. As expected, the thyroid deficiency produced by thiouracil caused a significant increase in pituitary weights (Table 2). No increase resulted from radiation alone, even though the nuclear changes in the thyroid following the larger doses of radiation were pronounced (Table 2).

In our original experiments (1) in which larger doses of radiation were delivered to the thyroid, there appeared to be an increase in the height of the epithelium. This has been confirmed by Rugh (28), Doniach and Logothetopoulos (7), Potter, Taurog and Chaikoff (8), and Maloof (6); however, only Doniach and Logothetopoulos used precise measurement to demonstrate such an increase. Since the mean cell height is a critical indi-

cator of thyroid stimulation, it was thought that subtle physiologic damage to the thyroid (as may have been caused by the small doses of radiation) might be reflected in an increase in cell height. In Table 4 are shown the mean cell heights, based on the measurement of 100 cells in each thyroid in the second series of animals after twenty-seven weeks. Thiouracil caused

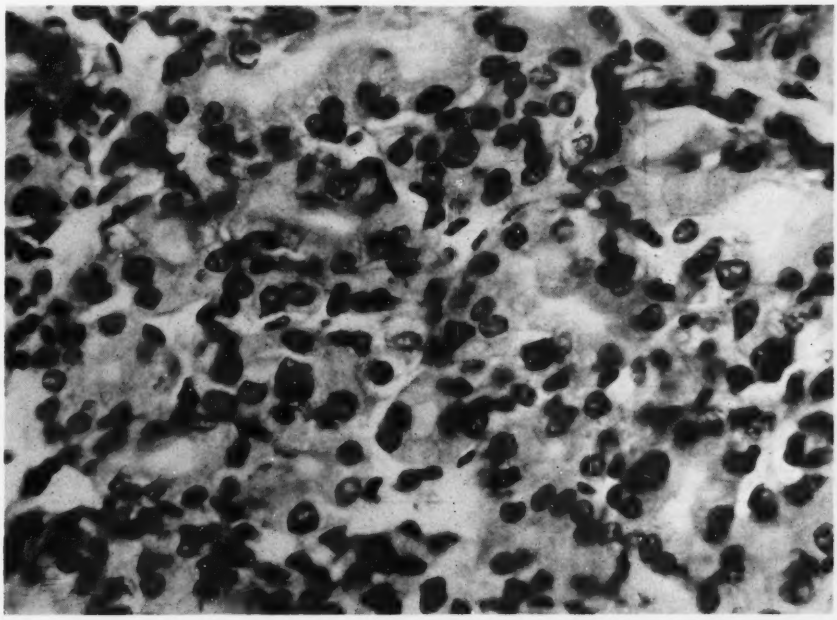

FIG. 4. Microscopic section of a representative thyroid on which DNA measurements were made.

This animal was from the second series and received 40 μc. of I^{131} followed by thiouracil. The preparation was stained with hematoxylin and eosin. Large hyperchromatic nuclei are evident.

an obvious increase in cell height. Furthermore this response could be produced to the same degree after the larger doses of radiation. Among the animals which had received only radiation, there was no significant difference in cell heights in relation to the increments in I^{131} dosage in this group; but, considering the series as a whole, a trend toward an increase in cell height was shown. Presumably the increase in cell height was a manifestation of the stimulus that caused the bizarre nuclear forms to appear (without thiouracil) in animals that had received radioiodine in amounts greater than 5 μc.

During the course of these experiments 3 thyroid neoplasms were found.

TABLE 4. MEAN CELL HEIGHT IN THE THYROIDS OF 3 ANIMALS FROM EACH GROUP SURVIVING FOR 27 WEEKS FOLLOWING RADIATION (SERIES II, TABLE 2)

Dose of I^{131} (μc.)	With thiouracil		Without thiouracil	
	Rat		Rat	
0	(#132)	11.25 ± 1.87	(#136)	6.67 ± 1.64
	(#134)	9.42 ± 1.50	(#138)	8.84 ± 1.96
	(#135)	10.69 ± 1.58	(#139)	6.80 ± 1.38
	Avge.	*10.45 ± 1.65*	*Avge.*	*7.44 ± 1.66*
5	(#142)	9.15 ± 2.01	(#144)	6.74 ± 1.55
	(#140)	9.74 ± 1.62	(#146)	7.94 ± 1.54
	(#143)	9.38 ± 1.75	(#147)	6.80 ± 1.48
	Avge.	*9.42 ± 1.79*	*Avge.*	*7.16 ± 1.52*
10	(#148)	10.93 ± 2.17	(#152)	6.92 ± 1.65
	(#149)	10.24 ± 1.72	(#154)	8.16 ± 1.57
	(#151)	11.82 ± 2.01	(#153)	7.94 ± 1.51
	Avge.	*11.00 ± 1.97*	*Avge.*	*7.67 ± 1.58*
20	(#156)	13.23 ± 2.05	(#162)	6.40 ± 1.05
	(#158)	10.79 ± 1.84	(#160)	8.78 ± 1.62
	(#159)	10.85 ± 1.98	(#161)	9.30 ± 1.75
	Avge.	*11.62 ± 1.96*	*Avge.*	*8.16 ± 1.47*
40	(#167)	12.38 ± 1.84	(#171)	9.47 ± 2.05
	(#164)	11.18 ± 1.60	(#168)	9.42 ± 2.08
	(#165)	11.33 ± 1.94	(#169)	9.45 ± 1.96
	Avge.	*11.63 ± 1.76*	*Avge.*	*9.45 ± 2.03*

All occurred in animals which had received only 5 μc. or 10 μc. of I^{131} and had subsequently been given thiouracil. The variation in nuclear DNA in these thyroids was not significantly different from that in the thyroids of other similarly treated animals in which tumors had not developed.

DISCUSSION

Originally some question was raised concerning the low DNA values observed in the thyroids of the seemingly normal rats. Although it is improper to draw comparisons between arbitrary units in different experiments or in different laboratories, the use of identical methods should yield results that are somewhat similar. The amount of nuclear DNA in the thyroids described here is low—approximately 1.3 arbitrary units per resting thyroid nucleus. Swift (19) and Leuchtenberger, Vendrely and Vendrely (20) reported values of 3.3 units for the liver and some other organs of the rat. The Feulgen staining of tissues in the first experiment was carried out by the personnel in Dr. Cecilie Leuchtenberger's laboratory at this University. Microphotometric measurements in both laboratories yielded values that were lower than expected for the normal thyroid tissues

in these animals. A review of the literature at that time furnished no information on the nuclear DNA content of thyroid cells. Because this perplexing problem of low DNA in the thyroid cells seemed to contradict established evidence that the cellular DNA content for different tissues was constant for the same species, further study of the staining reaction was indicated.

It was well known that insufficient hydrolysis on one hand, or prolonged hydrolysis on the other, results in less than maximal binding of the stain. Ris and Mirsky (29) had shown that a hydrolysis time of twelve to thirteen minutes was optimal in the liver. In the belief that the hydrolysis time used here for the thyroid might not have been optimal, a series of experiments on thyroid tissues was designed to test the staining intensity after varying periods of hydrolysis. It was found that the hydrolysis time of twelve minutes was optimal. Variation in hydrolysis was therefore not the cause of the low DNA values. Roels (30) has subsequently reported that the DNA in the thyroid cells of the rat is somewhat below the theoretical diploid value for this species.

The possibility was considered that the nuclei of the liver cells of these rats might contain more DNA than those of the thyroid. This hypothesis was tested by making a comparison of the nuclear DNA contents of the liver and thyroid cells of the rats used in these experiments. At the time the animals were sacrificed, some liver tissue was simultaneously fixed and processed along with the thyroid tissue. Microspectrophotometric measurements in both these tissues were then compared. The relative DNA values for the nuclei of the liver were quite similar to those for the resting normal thyroid. The low values obtained for thyroidal DNA, while they remain unexplained, do not invalidate the data pertaining to the effects of thiouracil and radiation, because parallel control observations were made.

It is generally believed that there is a fairly strict relationship between DNA and chromosomes, and that different tissues of the same species contain the same amount of DNA. However, there is some doubt that the DNA content is truly a rigid value. After much of this investigation had been completed, Alfert, Bern and Kahn (31) and Roels (30, 32) reported their studies on the relationship of change in physiologic activity to the DNA content of cells. They used propylthiouracil to stimulate and thyroxine to suppress the thyroid, and showed that the former increased DNA and the latter decreased it. The observations with thiouracil in the present study (see data on non-radiated animals in Figs. 1 and 2) add support to their findings.

It should be emphasized that chemical measurement of DNA in heterogeneous tissue such as the thyroid or even in homogeneous tissue, or estimation of nuclear DNA content as an average of many individual nuclear

measurements, does not furnish a true picture of the circumstances in the nuclei of a specific thyroid. The theoretical diploid value is based on an average of DNA values for nuclei which are presumably in a resting state; however, DNA may vary widely, depending upon the functioning state of the cells. Thus, although the mean DNA content of nuclei was calculated for the thyroids studied here, the standard deviations of the means for each animal are more revealing. For reasons of space, the average for a group of animals rather than the individual standard deviations was used in Tables 1 and 2. It was found that the observed changes were best illustrated by scatter graphs showing actual measurement of the DNA in individual nuclei. The first animal in each series was used in Figures 1 and 2 to illustrate the changes. With rapid multiplication of cells, the DNA seemed to be increasing in many of the nuclei, preparatory to division; however, amounts of nuclear DNA greater than twice the diploid value were extremely rare in the thyroid cells of non-radiated animals under the influence of thiouracil.

It is well known that some tissues, such as the liver, have nuclei in which the DNA occurs in multiples of the common value of DNA. Although Fautrez (33) reported that only one nuclear class could be found in the thyroid, the possibility that multiple classes of nuclei might occur in the thyroid must be raised. Walker and Yates (34) and Roels (32) showed that the DNA content of the nucleus increases rapidly just before cell division occurs. This increase is clearly shown in the animals given thiouracil. Roels (30) and Alfert, Bern and Kahn (31) observed an occasional thyroid nucleus with a tetraploid value in a normal gland. Under the influence of propylthiouracil, the frequency of these forms was increased, along with some intermediate values; however, there was no increase in the quantities of DNA, presumably because cellular division had occurred when the tetraploid value was reached. In the experiments of the present study, DNA was not observed above the tetraploid value until radiation had been introduced. The administration of even slight amounts of radiation resulted in some DNA values that were greater than twice the diploid value. This indicated that processes preparatory to cell division had progressed, but that actual division sometimes failed to occur. Failure or impaired ability of the gland to hypertrophy was observed in the same thyroids in which cell division seemed to have been thwarted.

If nuclei contain increased amounts of DNA just before cell division and presumably pass through a resting period before the next division, then the large nuclei found in radiated glands might represent different classes of nuclei. In some of the many individual scatter graphs prepared in this study, the distribution of DNA values suggested classes of nuclei containing amounts occasionally as high as eight times the diploid value. The

abundance of intermediate DNA values, in addition to casting doubt as to the presence of classes of nuclei, raised the question of whether the radiation had caused the loss of some chromosomes. The relationship between chromosomes and DNA has been demonstrated in many tissues, according to Alfert and Swift (35). After exposure to radiation a decrease in DNA may occur in some nuclei, as described by Warren, Holt and Sommers (36). The prevalence of intermediate amounts of DNA may have indicated that chromosomes had been destroyed and that subsequent chromosomal multiplication produced nuclei which did not lie within the range of one of the classes, as seen in the normal liver. Thus the many intermediate forms in the radiated thyroids probably represent chromosomal aberrations rather than multiple classes of nuclei. Even though micronuclei were observed in radiated thyroids in the present study, there was an extremely low incidence of DNA values that were significantly less than the diploid value.

A direct linear relationship between the volume and the DNA content of a nucleus was demonstrated by Swift (19) and by Alfert, Bern and Kahn (31); however, Alfert and associates did not believe that this linear relationship held true in the thyroid when a goitrogen was given. It is well known that the nucleus of the thyroid cell is relatively flat when in a resting state but becomes spherical when stimulated. In the studies described here (Figs. 1 and 2) the increase in the volume and the DNA content of the nuclei occurred in a roughly linear fashion; however, above the tetraploid value for DNA the relationship was less evident.

Potter, Taurog and Chaikoff (8) believed that the cellular hypertrophy produced in the radiated thyroid by the administration of propylthiouracil was not as great as that produced with this drug in a normal gland, although they did not document the degrees of hypertrophy with measurements. It is possible that the amount of radiation delivered to the thyroids of their rats was greater than that delivered in our study. The dose we used was sufficient to produce latent changes, but by actual measurement of the cells it was shown that the cell-height response in these radiated glands was equal to that produced in a normal gland.

In the present experiments it was observed that a few months after relatively low doses of I^{131} there might be no increase in DNA, but when more time was allowed to elapse following the radiation, some increase in DNA occurred. Since the stimulus of thiouracil caused the increase in DNA to appear soon after radiation, and since the increase in DNA was found to occur spontaneously after a greater interval of time, it was assumed that a deficiency of thyroid hormone played a part in the increase. Perhaps this situation in animals may be compared to that in human subjects treated with I^{131} for hyperthyroidism. In such patients a decline in metabolism often continues for a year or more after all I^{131} has disappeared from the

gland. The cells may linger, crippled but still functioning, and for a time may contribute to the thyroid hormone pool.

The disturbed cell division and abnormal chromosomal multiplication observed in the thyroid damaged by I^{131} result in abnormally high amounts of DNA—above the tetraploid value—which are not otherwise found in the thyroid. No conclusions can be drawn as to whether the irregular quantities of DNA described here have any relationship to the irregular quantities of DNA demonstrated to be present in malignant cells by Swift (14), Stowell (16), and Leuchtenberger, Leuchtenberger and Davis (17).

Acknowledgment

The authors are grateful to Miss Alice Wright, Miss Mary Ann Jenkins, Miss Carolyn Rolsten, and Mrs. Sheila Shear for assistance in the preparation of this manuscript.

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LONG-TERM EFFECTS OF HUMAN GROWTH HORMONE (LI) IN A PITUITARY DWARF*

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ABSTRACT

Human growth hormone (Li) was administered for one year to a pituitary dwarf between the ages of $11\frac{1}{2}$ and $12\frac{1}{2}$ years. The optimal dosage seemed to be 2.5 mg. daily intramuscularly; less frequent injections, even of the same total amount of hormone, were not as effective. An increase in height of 11 cm. ($4\frac{5}{8}$ inches) occurred during the year, compared to a previous yearly rate of 3 cm. ($1\frac{3}{4}$ inches) while the patient was taking sodium L-thyroxine. The fasting blood glucose level did not change, even though the glucose tolerance curve rose to mild diabetic levels during early metabolic studies while the patient was receiving growth hormone in doses up to 10 mg. daily. Later, the curve returned to normal levels during long-term administration of smaller daily doses. During a control period of seven months after the one year of treatment, growth slowed markedly. Undue bony maturation did not occur during or after treatment. When therapy was reinstituted, metabolic changes similar to those noted initially again occurred. The output of adrenocortical hormones tended to increase slightly during treatment. Urinary gonadotropin values rose during treatment, but there is some doubt as to the specificity of the test under these conditions. Psychologic evaluation showed no change in the patient's intellectual and emotional status.

METABOLIC studies with human growth hormone in a patient with pituitary infantilism have been previously reported by us (1). The present study describes the effects of long-term treatment on linear growth and skeletal maturation. The treatment period was one year, followed by a control period of seven months, after which therapy was resumed. Fur-

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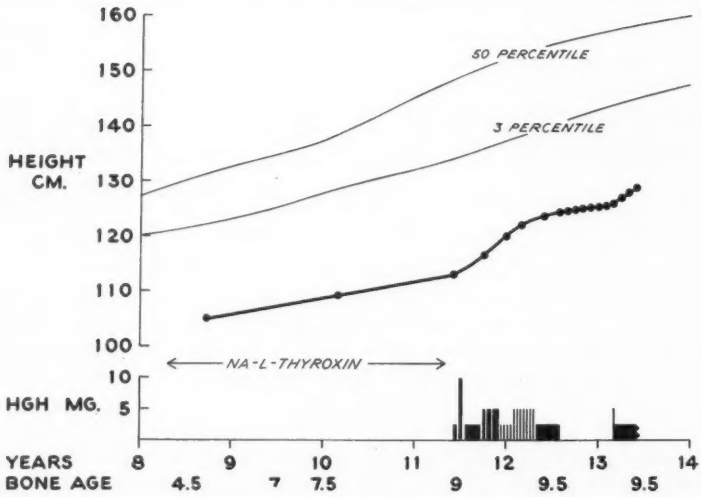

FIG. 1. Growth curve and bone maturation during treatment with sodium L-thyroxine and human growth hormone. Note increased rate of growth during treatment with human growth hormone and leveling of curve during control period. Bone age increased by less than one year during a year of treatment.

ther metabolic observations were made at intervals, together with estimations of function of other endocrine glands and also of the psychologic status of the patient.

CASE REPORT

C.B. (UCSF239441), a female, was aged 11 years and 5 months when the present study was started (1). Heights of her family were within the normal range and the patient had grown normally until age 6 months, when she had a febrile illness which apparently was encephalitis. Thereafter, her rate of growth and development was greatly slowed. At the age of 5 years, the bone age was 2 years. At the age of $8\frac{3}{4}$ years, her height was 105 cm. ($41\frac{1}{2}$ inches) (normal average, 132 cm. or 52 inches), and the bone age was $4\frac{1}{2}$ years. Studies at that time suggested borderline hypothyroidism, and sodium L-thyroxine was administered for two and a half years. Growth resulted, but at a slow rate, although the bone age advanced from $4\frac{1}{2}$ to 9 years (Fig. 1). At the age of 11 years and 5 months, when this study was begun, the girl's height was 113 cm. ($44\frac{1}{2}$ inches) (average for age, 149 cm. or $58\frac{3}{4}$ inches) (Fig. 2A), and the bone age was 9 years. Metabolic balance studies, as previously reported (1), showed nitrogen, sodium, potassium and phosphorus retention. Serum alkaline phosphatase activity was slightly elevated. Calcium diuresis occurred initially, but was followed by retention; the glucose tolerance curve became higher, although the fasting blood sugar level was not affected. After these studies, on February 21, 1958, long-term therapy was started with human growth hormone[1] administered in dosages of 2.5 mg. daily for two months, 5 mg. daily for two

[1] The human growth hormone was prepared by the method of Li (2, 3), and sterilization was accomplished by passing the solution through a B-D Swinny filter adapter using a millipore filter, type HA, 0.45 μ.

months, 2.5 mg. every second day for two months, 5 mg. every second day for three months, then 2.5 mg. daily for three months.

After one year of continuous treatment, human serum albumin (Cutter Laboratories) was substituted as placebo therapy for three months. Next, a three-month vacation from all injections was allowed, after which the patient was readmitted to the hospital for study. Continuous therapy was reinstituted approximately one month later, following a total of seven months without treatment. Estimations of bone age were made periodically. Formal psychologic testing of intellectual functioning and emotional status was carried out at intervals by Dr. Helen Gofman. During the year of treatment with growth hormone, the girl's height increased considerably (Fig. 2B). The growth curve and bone age values are shown in Figure 1. Growth tended to occur in spurts, so each dosage level was tried for a period of two or three months; 2.5 mg. daily intramuscularly seemed most effective. The rate of growth approximated 1 cm. per month, totaling 11 cm. ($4\frac{3}{8}$ inches) in one year. The previous rate of growth during administration of sodium L-thyroxine had been 3 cm. per year. During the seven-month control period, the rate was slower (Fig. 2C). Resumption of treatment with 2.5 mg. daily of human growth hormone resulted in a return to a growth rate of approximately 1 cm. per month. During the nineteenth-month period of observation, the bone age had advanced less than one year.

No hyperglycemia or glycosuria occurred during long-term treatment, and the glucose tolerance curve returned to control levels during treatment (Fig. 3). After resumption of therapy, retention of nitrogen, sodium, potassium and phosphorus again was demon-

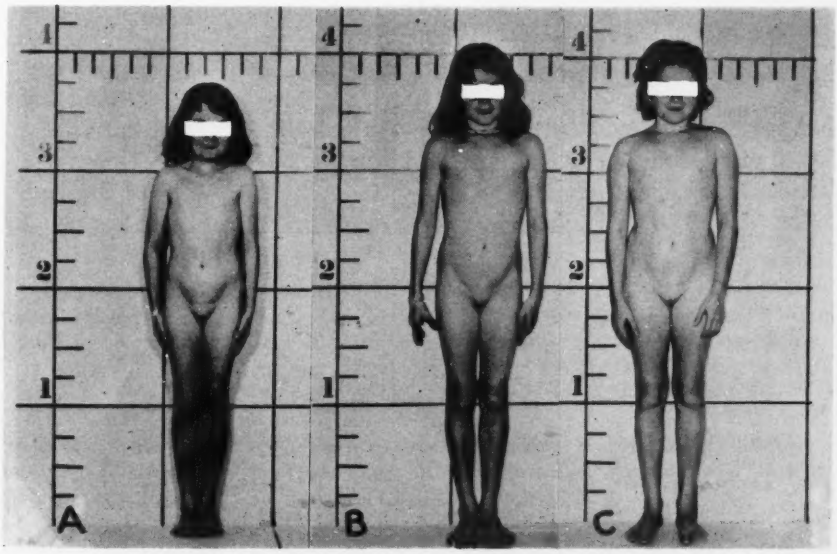

FIG. 2. The patient: **A**—on January 3, 1958, *before therapy*, age 11 years and 6 months, height 113 cm. ($44\frac{1}{2}$ inches), weight 18.4 Kg. ($40\frac{1}{2}$ pounds). **B**—on February 17, 1959, *after one year of continuous therapy*, age 12 years and 7 months, height 125 cm. ($49\frac{1}{4}$ inches), weight 23.5 Kg. ($51\frac{3}{4}$ pounds). **C**—on September 25, 1959, *after a seven-month control period*, age 13 years and 2 months, height 126 cm. ($49\frac{3}{8}$ inches), weight 25.5 Kg. (56 pounds).

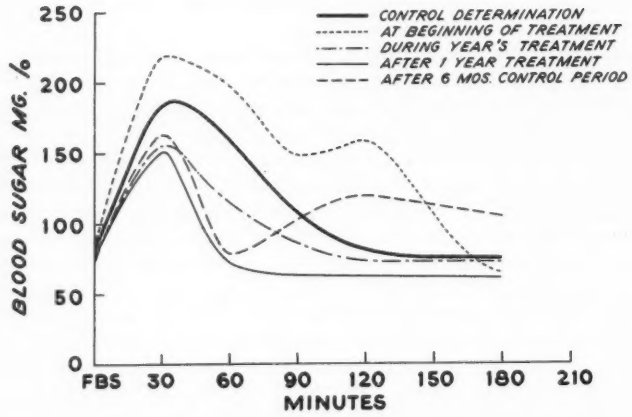

FIG. 3. Glucose tolerance curves. Note higher and more prolonged curves at beginning of treatment period, followed by return to normal levels.

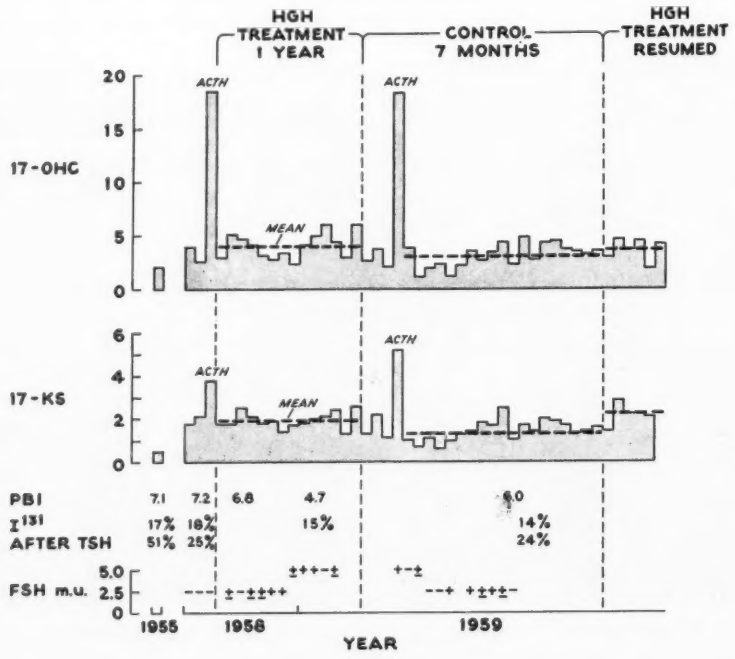

FIG. 4. Assessment of endocrine function during 19-month period of observation.

strated. Calcium diuresis and a prompt fall in the level of blood urea nitrogen occurred. Some elevation of the serum alkaline phosphatase level again was noted.

Assessment of various endocrine functions (Fig. 4) showed no change in that of the thyroid. Urinary excretion of 17-ketosteroids and 17-hydroxycorticoids rose slightly during therapy; urinary aldosterone levels measured by a double isotope-labeling technique (4) were not affected. Urinary gonadotropin determinations[2] showed some increase during treatment and a fall to previous levels during the control period. The possibility of a direct effect of the growth hormone on the mouse uterus during tests for gonadotropin excretion was considered as an explanation for the higher levels during therapy.

Interviews for appraisal of emotional status showed an elevation in mood during treatment when the rate of growth was measured. No immediate changes could be observed when placebo injections were substituted. The level of intellectual function was not changed.

DISCUSSION

The causal relationship between human growth hormone therapy and the increase in the rate of linear growth in this patient seems well established, and agrees with the reported findings of Raben (6) and Henneman and Henneman (7). It is especially convincing in view of the pronounced diminution in the growth rate during the placebo and no-treatment periods. Moreover, the growth response seems outstanding in that it was not accompanied by undue bony maturation, either during or following the period of therapy. Since the rate of increase in bone age can vary considerably from year to year even in normal children, further observations on this patient will be of interest.

Long-term administration of this growth hormone preparation has thus far produced no toxicity. The fluid retention and the tendency to hyperglycemia noted in the previous study on this patient (1) did not recur with the dosage used.

Acknowledgments

We again thank our colleagues in various departments of pathology who collected pituitaries for use in this study: Drs. Robert R. Wright, John W. Swift, John H. Powers and Pierce A. Rooney of the Herbert C. Moffitt Hospital of the University of California; Dr. Arthur R. Lack of the San Mateo Community Health Center; Dr. Harlow D. Standage of the Santa Clara County Hospital; and Colonel Nelson S. Irey (MC) of the Letterman Army Hospital, San Francisco.

A grant from the Jefferson-McLeod-Moore Fund made possible the establishment of a Pituitary Bank at this center as a source of pituitaries for extraction of growth hormone for use in this and future studies.

The study would not have been possible without the help of the laboratory and dietary workers of the Metabolic Unit for Research in Arthritis and Allied Diseases and the Pediatric Microchemical Laboratory of the University of California Medical

[2] Measured by the method of kaolin adsorption and acetone extraction, with an increase in uterine weight in immature 21-day-old Swiss albino mice as the end-point (5).

Center. We also wish to thank Dr. Eugene Eisenberg for assistance with calcium determinations and Dr. E. Biglieri for urinary aldosterone determinations.

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Letters to the Editor

MALE PSEUDOHERMAPHRODISM WITH FEMALE CHROMOSOMAL COMPLEMENT

To the Editor:

Since the experience of most investigators (1) has been that all the variants of male pseudohermaphrodism are genetic males, we should like to report a case that seems to fit the clinical diagnosis of male pseudohermaphrodism but with evidence of the female chromosomal complement—a new type of human intersex. Detailed clinical, endocrinologic and cytologic studies are presented.

CASE REPORT

Mr. S.P., aged 20 years, was referred to us because of ambiguous external genitalia. The family history (*e.g.*, the mother's age, and health during pregnancy) was noncontributory. The patient had suffered from no other serious illness except cerebral malaria at the age of 2 years and typhoid at the age of 3 years. On the whole, he had enjoyed general good health, though his physical growth and mental development had been always below par. On the basis of his intelligence quotient, he could be classified as an idiot.

The patient was slightly built, poorly nourished, and had a pigeon-shaped chest. He had no facial or axillary hair, and the suprapubic hair was sparse and of feminine distribution. He weighed 75 pounds, was 60 inches tall, and had eunuchoidal skeletal proportions. In the left inguinal canal there was a bulging, reducible round mass, 3 cm. in diameter. This mass proved to be the left testis and epididymis.

The external genitalia appeared hermaphroditic and consisted of an enlarged phallus, two skin folds with scrotal rugae and pigmentation, but no palpable gonads. When these folds were parted, a vestibulum could be seen in which there were separate urethral and vaginal openings. The phallus was 4.5 cm. in length and 6.5 cm. in circumference. The vaginal canal was 7.5 cm. long. On rectal examination no midline structure (*e.g.*, prostate or uterus) was palpable. The rest of the physical findings were unremarkable.

Urine and blood analyses (including hemoglobin level) yielded normal results. In repeated vaginal smears there were a few precornified squamous cells with large groups of endometrical cells. A skiagram of the pelvis following injection of radio-opaque material through the vagina showed a vaginal canal with a thin tract extending into the right pelvic cavity, suggesting a fistulous remnant of the Müllerian duct.

Urinary gonadotropins were increased (positive titer at 100 m.u. per twenty-four hours). Urinary 17-ketosteroid excretion was low on repeated estimation, whereas urinary ketogenic steroids were normal. The level of plasma corticosteroids was normal. Further details will be published elsewhere.

The findings at laparotomy are shown in Figure 1. On each side was a gonad macroscopically resembling a testis. Similarly, structures resembling an epididymis and vas deferens could be seen on each side. The broad ligament was present, but no mass resembling uterus could be seen or palpated. The structures shown in the areas demarcated within the dark circles (Fig. 1) were removed and subjected to histologic examination. Each gonad measured 2.0 cm. in length, 1 cm. in breadth, and 0.8 cm. in thickness.

Serial histologic sections of the gonads stained with haematoxylin and eosin showed a number of immature seminiferous tubules, some areas of hyalinized tubules, and masses of interstitial cells. The tubules were lined by a single layer of undifferentiated cells. The Leydig cells were aggregated in a large, well vascularized mass. The individual

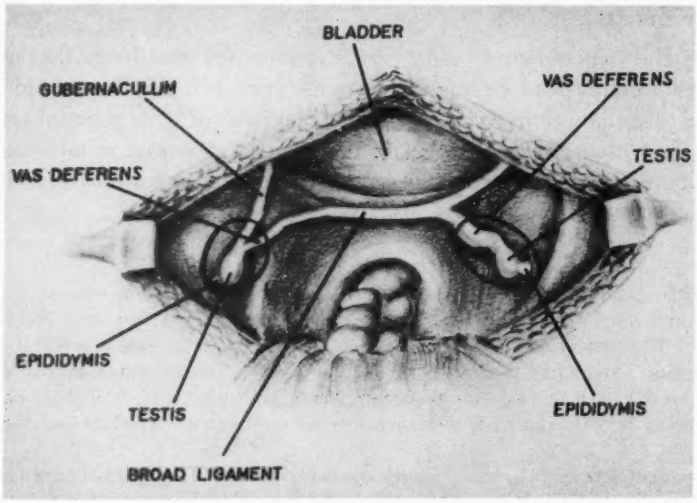

FIG. 1. The operative findings.

Leydig cells had vesicular nuclei with eosinophilic cytoplasm containing fine granules (Fig. 2). In the sections of the testes stained with Mallory's connective tissue stain there was a mild degree of tubular fibrosis, and in the sections treated with Weigert's stain there was total absence of elastic fibers in the tubular wall of the seminiferous tubules. No ovarian elements were seen in any of the sections of the gonads.

Microscopic examination of the organs resembling epididymis and vas deferens on both sides demonstrated the typical structure of these tissues.

Cytologic examination showed that the nuclei of the Leydig cells of the testes and also of the cells from the buccal mucosa, the skin and the vaginal epithelium contained a normal female proportion of sex chromatin bodies. The sex chromatin bodies were typical and were stained by the modified Feulgen technique (2).

The chromosomal study of this case was made by Dr. D. G. Harnden of the Western General Hospital, Edinburgh. A skin biopsy specimen was sent from Bombay to Edinburgh and a culture was established from this tissue by the method of Harnden (3).

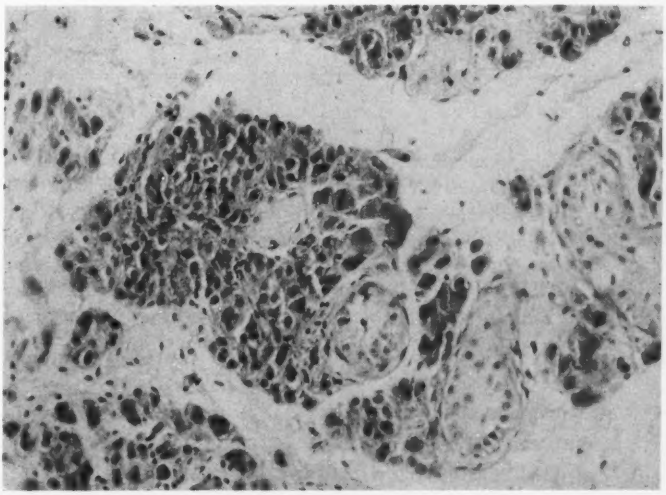

FIG. 2. Histologic section from the right testis (reduced from H & E, ×180); the sections from the left gonad were identical in microscopic appearance.

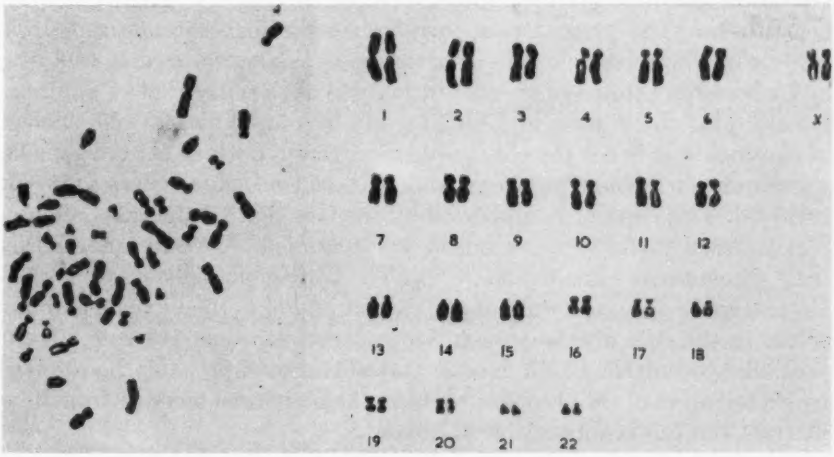

FIG. 3. Mitotic metaphase and arrangement of chromosomes in pairs. The ideogram shows 46 chromosomes, XX sex chromosomal complement.

TABLE 1. CHROMOSOME COUNTS IN CELLS OF SKIN BIOPSY SPECIMEN

Chromosome count					Total cells	
<45	45	46	47	>48	counted	analyzed
1	2	27	—	—	30	3

The chromosomes were 46 in number and indistinguishable from those of a normal female, *i.e.*, they had an apparently XX sex chromosome constitution (Table 1 and Fig. 3). The few cells with other than 46 chromosomes were considered to be the result of technical errors, and the cells with a count of 47 chromsomes could not have been interpreted as having an XXY sex chromosome constitution. A second biopsy specimen was sent later in order to check these findings and to exclude the possibility that this may have been a case of XX/XXY mosaic. Unfortunately, upon culture, this specimen failed to grow.

COMMENT

Recent data indicate that Klinefelter's syndrome, which was thought at one time to be an example of complete sex reversal in the chromatin-positive subject, is associated with an abnormal complement of sex chromosomes (4). The only apparent exception is the case reported by Ford, Jacobs and Lajtha to have 46 chromosomes and an XX sex chromosome constitution. This finding was based on the examination of only 5 cells, and is therefore not conclusive. All the other reported cases of chromatin-positive Klinefelter's syndrome appear to have had an XXY sex chromosome constitution. The present case therefore is the first chromatin-positive subject in whom both gonads, on detailed sectioning, proved to be testes and whose sex chromosome constitution was apparently that of a normal female. This observation in itself suggests that an abnormal complement of chromosomes is not the only mechanism through which altered gonadal differentiation is brought about, and that several causal factors may be involved. This concept is supported by the fact that all the cases of chromatin-positive true hermaphrodism so far studied have had a normal female chromosome constitution (6–10). The finding of a female sex chromatin pattern in various tissues of our patient and of normal female chromosomes in the cells of a small skin biopsy specimen does not rule out the possibility of an XX/XXY mosaic (11). This could probably be resolved by examination of the chromosome from the peripheral blood or from bone marrow, but this is not possible at present.

If by further study we should exclude this possibility, two interpretations of the altered gonadogenesis would be consistent with the finding of an XX sex chromosome complement: 1) the sex reversal could have been effected by an autosomal recessive gene mutation such as the one described in Drosophila by Sturtevant (12); or 2) deleterious environmental factors could have destroyed the cortex at the time of onset of cortico-medullary

competition. If either of these explanations be correct, the present case may be only a partial expression of the phenomenon, and eventually a normal female chromosome complement may be found in a case clinically indistinguishable from chromatin-positive Klinefelter's syndrome.

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REMARKS CONCERNING TWO TYPES OF BASOPHIL CELLS IN THE HUMAN ADENOHYPOPHYSIS*

To the Editor:

Two types of basophil cells have been tinctorially distinguished in the human pituitary by a variety of methods: the kresazan or resorcin fuchsin-azan technique of Romeis (1), postchromation followed by staining with periodic acid-Schiff (PAS)-orange G-hematoxylin, or with aldehyde fuchsin, or with the Cleveland-Wolfe method (2), performic acid-alcian blue-PAS-orange G (3), performic acid-aldehyde fuchsin (4, 5), PAS-methyl blue (6), iron-PAS (7), and others (7, 8). Adams and Swettenham (3) called the *granules* which stained blue with the performic acid-alcian blue-PAS technique, S granules, and those which were tinged red, R granules. The two types of granules may occur within the same cell. Nevertheless, results with Adams and Swettenham's technique have been included in tabulations representing attempts to correlate basophil *cell* types distinguished by the various methods (7, 8). Furthermore, since these attempts were based partly on conjecture rather than on staining of adjacent sections by the different techniques, the relation of the two basophil cell types differentiated by various authors is far from clear, and the equation of such cells with the beta and delta cells described by Romeis in his classical work (1) is hazardous or unwarranted. The confusion which has resulted from the extension of Romeis' nomenclature to other species has been discussed (8, 9).

In human basophil cells the S granules are said to represent the cystine-rich protein which is present in crude ACTH preparations (3, 8). On the other hand, Russfield (5) suggested that the cells which stain with aldehyde fuchsin after performic-acid treatment are the same as those which contain S granules, and should therefore correspond to the delta cells reported by Ezrin *et al.* (7), the absence of which in childhood and pregnancy led to the conclusion that they are the source of gonadotropin (10). Russfield's bioassays of pituitaries in which the estimated number of aldehyde-fuchsin positive cells was correlated with the capacity of the glands to cause an increase in ovarian weight are in poor agreement with this interpretation, since pituitary glands devoid of such cells also stimulated ovarian growth. Herlant (2) considered the cells he called "delta" to be the source of thyrotropin, since they were abundant in the hypophysis of a thyrotoxic patient, and since he believed that they give rise to the large, partially degranulated cells containing many colloid droplets which often

* Aided by USPHS grant A-2463.

abound in the pituitaries of myxedematous subjects (11). Ezrin *et al.* (11), however, suggested that these cells are derived from what they call beta cells. These quotations from the literature amply illustrate the confusion which exists regarding the function of human pituitary basophil cell types.

A new technique, aldehyde-thionin-PAS, has been described as differentiating the two types of basophil cells in dog and rat pituitaries fixed in formol-sublimate (12, 13). In the rat pituitary, aldehyde-thionin stains the thyrotrophs but also demonstrates coarse droplets in the PAS-positive gonadotrophs (personal observation). We have found that aldehyde-thionin stains the granules blue in some human pituitary basophils, and PAS stains the granules a reddish color in others. We have used this technique on sections from 3 formol-fixed human pituitaries—one from a 73-year-old man, the second from an 11-year-old boy, and the third (received from Dr. Ezrin) from a 31-year-old man. Nuclei were stained with hemalum, and acidophils with orange G. Adjacent sections were stained by Dr. Ezrin, to whom we are indebted for this favor, with the iron-PAS technique; in the case of the first two glands, sections next to these were stained with performic acid-alcian blue-PAS-orange.

Basophil cells in which the granules were aldehyde-thionin positive were found in all 3 pituitaries. They outnumbered the PAS-stained basophils in the pituitary of the 73-year-old man, were about half as numerous as the latter in the pituitary of the young man, and were least abundant (but still numbering scores per horizontal section of the anterior lobe) in the pituitary of the child. These aldehyde-thionin positive cells were clearly not identical with Ezrin's delta cells, since he found no delta cells in the pituitary of the old man, very few in that of the child, and as many delta cells as beta cells in that of the young man. Aldehyde-thionin positive cells and cells with S granules seemed to be in better numerical agreement, but in the hypophysis of the old man many cells contained both S and R granules, and such transitions between aldehyde-thionin positive and PAS positive cells could not be clearly seen in this gland.

The cells which stained with aldehyde-thionin were usually smaller than those which were PAS positive, and contained fewer granules. They resembled Romeis' delta cells (1) in appearance. Intracellular colloid droplets in other cell types also stained with aldehyde-thionin, as did some intercellular colloid and the neurosecretory substance of the posterior lobe. Cells of the intermediate lobe which had invaded the posterior lobe were PAS positive and aldehyde-thionin negative.

Thus aldehyde-thionin-PAS is a clear and comparatively simple differential stain for two types of pituitary basophils in man. It is questionable whether it differentiates S from R granules, and it evidently does not distinguish between the same "beta" and "delta" cells as those distinguished

by the iron-PAS technique (7); however, it may set apart Romeis' beta and delta cells (1) as well as does the much more cumbersome kresazan method. The non-identity of Romeis' and Ezrin's delta cells, as implied in this statement, is attested to by the former's observation that his delta cells were present in *all* pituitaries, including those of pregnant women (1), unlike the delta cells of Ezrin. The histophysiologic evaluation of the aldehyde-thionin-PAS technique and its more extensive comparison with other methods may help in resolving the difficulties which at present beset the functional assessment of human adenohypophysial structure.

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† National Institutes of Health predoctoral fellow.

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EOSINOPHILIC ADENOMA OF THE PITUITARY AND CARCINOID TUMOR OF THE RECTO-SIGMOID AREA

To the Editor:

Attention was focused on the entity of multiple endocrine adenomata by Underdahl, Woolner and Black (1) in 1953. In the January 1960 issue of this Journal, Southren (2) described a patient with a pituitary tumor, acromegaly, and a concomitant functioning bronchial carcinoid without the clinical features of the carcinoid syndrome.

A similar case was observed by us recently, that is, adenoma of the pituitary with acromegaly and a carcinoid tumor of the recto-sigmoid area not accompanied by the "carcinoid syndrome."

The patient was a 38-year-old colored male. During the two years prior to admission the typical clinical features of acromegaly had developed. These included spade hands, large feet, an increase in head size with prognathism, galactorrhea, loss of libido, and generalized weakness. He had received x-ray irradiation to the pituitary area in another hospital, but continued to have headaches and considerable weakness. He also had simple glaucoma, and hypertension of moderate degree (blood pressure, 170/120 mm. Hg), which apparently antedated the acromegalic changes.

There was no loss of peripheral vision, though the sella turcica was distinctly enlarged. Pneumoencephalograms showed generalized dilatation of the lateral ventricles without displacement. The spinal fluid was under increased pressure 300 mm. of water) and the concentration of spinal fluid protein was 54 mg. per 100 ml.

The serum protein-bound iodine level was low (3.7 μg. per 100 ml.). The 24-hour urinary excretion of 17-ketosteroids was 0.4 mg. and that of 17-hydroxycorticoids was 3.6 mg. In spite of these low steroid levels, the patient's response to salt deprivation and to water loading was normal. The glucose tolerance test showed "probable diabetes" by the criterion of Conn; the blood sugar level rose in one hour to 161 mg. per 100 ml., and in two hours was 118 mg. per 100 ml. There was no glycosuria. The serum phosphorus level was 4.6 mg. per 100 ml.; otherwise, serum electrolyte concentrations, including that of calcium (10.8 mg. per 100 ml.), were in the normal range. The serum alkaline phosphatase level was 6.6 King-Armstrong units. The concentrations of blood epinephrine and norepinephrine were also normal, as determined fluorometrically by the ethylene-diamine condensation method (epinephrine, 0.9 μg. per liter; norepineph-

rine, 2.9 μg. per liter). There was no evidence of parathyroid or pancreatic islet-cell hyperfunction. The thyroid was moderately enlarged, but no nodules were felt.

The finding of carcinoid tumors of the recto-sigmoid was fortuitous. The patient was anemic and had a history of having passed bright red blood by way of the rectum. Sigmoidoscopy revealed two small polypoid lesions in the recto-sigmoid area, and biopsy of these showed typical carcinoid tumor (Figs. 1 and 2.)

There were no symptoms, except possibly the hypertension, to indicate that these carcinoids were functioning. During a period of banana-free diet the 24-hour urinary excretion of 5-hydroxy indole acetic acid was 7.6 mg.—normal for our laboratory.

The patient refused additional studies at that time. He has since returned to the clinic and has received further x-ray treatment to the pituitary area. Over a period of ten months, to date, there has been no evidence of progression of the carcinoid tumors.

Three previous case reports describe bronchial carcinoids associated with other endocrine adenomata (1–3). Two of these patients had acromegaly (2, 3). A functional relationship may exist between pituitary tissue and argentaffin tissue, since recent work indicates that a hyperglycemic

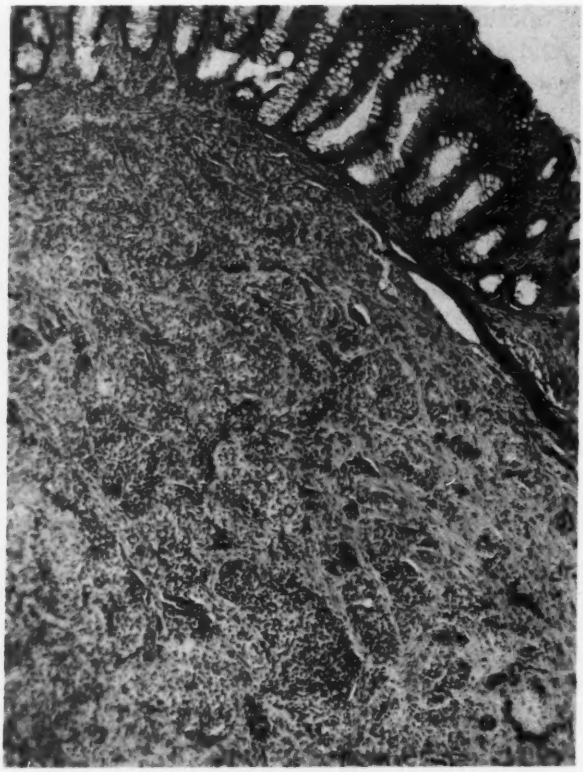

FIG. 1. Intact mucosa over carcinoid tumor. H. & E., ×50.

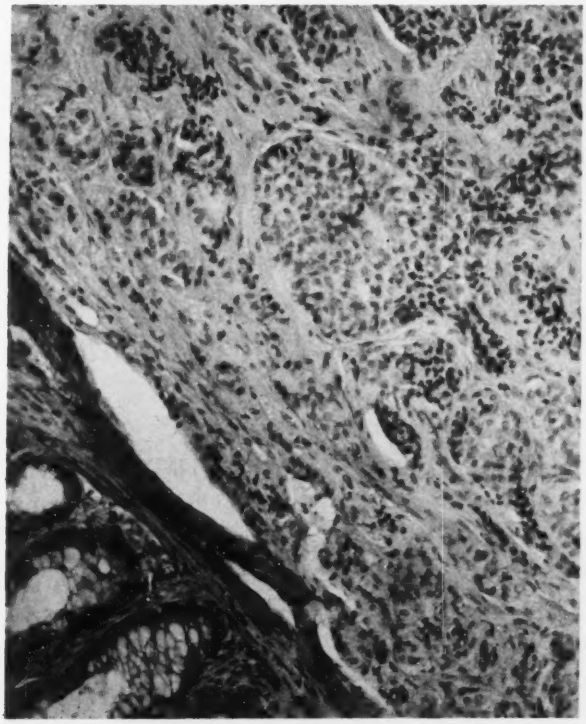

FIG. 2. Submucosal alveolated clusters of neoplastic cells with relatively uniform rounded nuclei. H. & E., ×150.

substance released from the pancreatoduodenal area under the influence of growth hormone may be serotonin (4, 5). A search for carcinoid tumors in acromegalic patients may be warranted.

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THYROTROPIN AND THYROIDITIS

To the Editor:

The recent article in this Journal by Danowski and associates (1) entitled "Thyroiditis Following Administration of Thyroid-Stimulating Hormone," records a useful observation of interest to many students of thyroid disease. Attention should be called, however, to what may prove an unfortunate semantic dilemma which adds confusion to the existing literature on thyroiditis.

The authors describe a striking and uncomfortable response in volunteers to a particular batch of exogenous thyrotropin. They classified the response as "thyroiditis" on clinical grounds, because the symptoms described could be interpreted as resulting from an inflammatory process in the thyroid; these symptoms were enlargement of the thyroid, local heat and "systemic prostration." That this assumption may be correct must be acknowledged, but it remains undocumented without histologic examination of the thyroids so affected. It is possible that, instead of reflecting inflammation, the symptoms might have reflected intense hyperemia and hypertrophy induced by the specific biologic properties of thyrotropin, accompanied by a nonspecific systemic response to foreign protein.

The term "acute thyroiditis," which these authors employ in the text and abstract of their article, unfortunately has long been pre-empted in the literature by a condition with characteristic clinical features and unique physiologic and histopathologic aspects. This condition, ordinarily known as "acute thyroiditis," but also known as "subacute thyroiditis," "granulomatous thyroiditis," or "de Quervain's disease," obviously differs in all important respects from that described by Danowski and associates. De Quervain's thyroiditis has, in fact, been treated effectively by administration of exogenous thyrotropin as well as by antithyroid drugs, adrenal steroids and other measures. Nevertheless, the exigencies of medical indexing and abstraction being what they are, there is little hope that the

condition described by Danowski and associates will be preserved for posterity under any subheading other than "acute thyroiditis," where it may forever be confused with a more familiar and wholly different disorder.

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January 6, 1961

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INHIBITION OF THYROIDAL I^{131} UPTAKE BY PARABROMDYLAMINE MALEATE

To the Editor:

It is well known that the administration of drugs containing iodine suppresses the thyroidal uptake of radioactive iodine. Not as widely recognized is the fact that various antihistamines have a similar effect in some patients (1).

The purpose of this communication is to report the effect of parabromdylamine maleate[1] on the thyroidal uptake of radioactive iodine (I^{131}) in untreated hyperthyroid and euthyroid patients.

MATERIALS AND METHODS

Twenty-two patients (8 females and 14 males) were studied. Thirteen were hospital patients and 9 were out-patients. The thyroidal uptake of I^{131} was determined after administration of a 10-microcurie dose, using a collimated 2.5×2.5 cm. thallium-activated sodium iodide crystal placed 25 cm. from the neck. Normal uptake values for this laboratory are 10 to 30 per cent of the dose in five hours and 15 to 50 per cent in twenty-four hours. Following the initial uptake measurements, parabromdylamine was administered orally in a dosage of 16 mg. daily to 5 patients, and 24 mg. daily to 17 patients, for a period of seven days. On the eighth day the I^{131} uptake was again determined.

RESULTS

Euthyroid group (Table 1). In 8 of 17 patients there was a reduction in I^{131} uptake; in 5 of these 8 the reduction in the 5-hour uptake was less than 10 per cent. In 7 patients there was reduction in the 24-hour uptake; 2 of these uptakes were below 15 per cent and 5 were low-normal. Nine patients showed no change in either the 5-hour or the 24-hour uptake.

[1] Supplied as Dimetane® by Dr. J. O. Burke, A. H. Robins Co., Inc., Richmond, Virginia.

TABLE 1. INHIBITION OF THYROIDAL I^{131} UPTAKE BY PARABROMDYLAMINE IN 8 EUTHYROID SUBJECTS

Subject No.	Control period		Parabromdylamine for seven days	
	5-hr. uptake %	24-hr. uptake %	5-hr. uptake %	24-hr. uptake %
1	11.3	—	7.0	—
2	14.1	24.6	8.4	16.2
3	13.7	23.5	6.6	15.1
4	17.0	28.0	9.0	15.5
5	10.0	24.4	6.0	14.0
6	—	23.0	—	15.0
7	—	28.0	—	15.2
8	—	16.9	—	11.9

Hyperthyroid group. In 2 of 5 patients there was a reduction in both the 5-hour and the 24-hour uptake to the normal level; in the other 3 there was no change. In the 2 patients whose uptakes were reduced to the normal level, there was a return to the pre-treatment values one week after cessation of parabromdylamine therapy.

DISCUSSION

The results in both groups are in agreement with those previously reported (1). The 2 hyperthyroid and the 8 euthyroid subjects in whom there was a reduction of thyroidal I^{131} uptake, did not manifest any side-effects from parabromdylamine. There was no significant difference between the degree of inhibition induced by the 16-mg. dosage and that induced by the 24-mg. dosage.

The data indicate that parabromdylamine therapy will inhibit the thyroidal uptake of I^{131} in some patients. This medication should be discontinued at least one week prior to the performance of an I^{131} uptake test.

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November 30, 1960

Acknowledgment

The technical assistance of Lt. Comdr. Virginia Donson, A. V. McKeon, H. M. Van Wagner, and M. V. Donahue is greatly appreciated.

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The Endocrine Society

The Forty-Third Annual Meeting

General Information

Date: Thursday, Friday and Saturday,
June 22–23–24, 1961

Headquarters Hotel Biltmore,
New York, N. Y.

Registration:

Fountain Room, Hotel Biltmore.
Everyone attending the meetings is required to register. By action of the Council, nonmembers will be charged a registration fee of $10.00 for any part of the scientific sessions.

Fellows, graduate students, residents and interns will be admitted upon presentation of proper credentials CERTIFIED BY DEPARTMENT HEAD OR SUPERINTENDENT OF HOSPITAL and payment of $2.00 registration fee.

Members should present their current membership cards when registering. Admission will be by badge only.

Your Endocrine Society badge will admit you to the American Diabetes Association's opening meeting Saturday morning at the Hotel Commodore. Members of the Endocrine Society are requested to register (without charge) at the desk of the American Diabetes Association.

Scientific Sessions:

All morning sessions will be held in the Ballroom. Simultaneous sessions will be held each afternoon in the Ballroom and the Bowman Room. Sessions will begin promptly on schedule. Presentation of all submitted papers will be LIMITED TO TEN MINUTES. All speakers will be required to adhere strictly to schedule. Speakers who have manuscripts for publication are invited to submit them to the Editor of ENDOCRINOLOGY or of THE JOURNAL of CLINICAL ENDOCRINOLOGY AND METABOLISM.

Annual Dinner:

The Annual Dinner of the Society will be held on Friday evening, June 23, at 7:30 o'clock in the Ballroom. Cocktails will precede the dinner in the Fountain Room at 6:30 P.M. Secure tickets at time of registration.

Local Arrangements:

Dr. Nicholas P. Christy is Local Chairman and Mrs. Warren O. Nelson is in charge of the Women's Entertainment Committee.

(For program, see the May issue of the Journal)

The 1961 Awards

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors. Endocrine Society awards are limited to citizens of the United States and Canada.

Fred Conrad Koch Award

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, Distinguished Service Professor of Physiological Chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is the highest honor of The Endocrine Society and is represented by a medal known as the Koch Medal of The Endocrine Society. The medal, as well as an honorarium of $3,500, is to be given annually to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

This Award was first presented in 1959, to Dr. W. W. Swingle and in 1960 was presented to Dr. Emil Witschi. The Koch Medal has replaced the Medal of The Endocrine Society which was established in 1954.

The Ciba Award

The Ciba Award is given in recognition of the meritorious accomplishments of an investigator in the field of basic or clinical endocrinology who has not reached his thirty-sixth birthday before June 1 of the year in which

the Award is presented. This Award was established in 1942 and first presented in 1944. The Award is presently in the amount of $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Scholar of the Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. A grant has been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and the Squibb Fellowships may be made by any member of

The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than *October 15* each year.

Proposals for appointments as a Scholar of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by *October 15* each year.

Thirteenth Postgraduate Assembly in Endocrinology and Metabolism

UNDER THE CO-SPONSORSHIP OF THE ENDOCRINE SOCIETY
AND THE NATIONAL INSTITUTES OF HEALTH
BETHESDA, MARYLAND
October 2–6, 1961

A comprehensive review of clinical endocrine problems and current research activity in these areas will be presented. For further information, write to: Dr. Roy Hertz, National Institutes of Health, Building 10, Bethesda 14, Maryland. The fee will be $100.00 for physicians, with a reduction to $30.00 for Residents and Fellows. Enrollment limited to 100.

* * * * *

American Goiter Association—Recipients of Awards

VAN METER PRIZE AWARD

In 1930, S. D. Van Meter, M.D., of Denver, a founding member of the American Goiter Association, offered a prize of $300.00 to be awarded to the essayist presenting the best work on the subject of "Goiter—Especially its Basic Cause." Doctor Van Meter enthusiastically supported the object of the Association, as given in the Constitution " . . . the acquisition and spread of knowledge of the thyroid gland and its diseases." Upon his death

in 1934, the Association continued the award and named it after its originator.

Throughout the years, essays have been received from many foreign countries as well as our own; competition has been keen, and the committee selected to review the manuscripts has frequently found it difficult to name a winner.

The winner of the Award this year was *James Bernard Field, M.D.* of Bethesda, Maryland. The Award was announced during the Annual Meeting of the American Goiter Association in the Warwick Hotel in Philadelphia. Doctor Field's winning manuscript was entitled, "Studies on the Mechanism of Thyroid Stimulating Hormone." He was assisted in this work by Ira Pastan, M.D.

Doctor Field received his Doctor of Medicine degree Cum Laude from Harvard Medical School in 1951. His postgraduate training began in the Massachusetts General Hospital, Boston, and continued in the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health. He is currently a senior surgeon of the U.S.P.H.S. in the Institute of Arthritis and Metabolic Disease.

Other honors have been presented to Doctor Field in the past. He is a member of the Honor Medical Fraternity Alpha Omega Alpha. He received the Harvard Veterans National Scholarship from 1946 to 1951. In 1951 he received the Massachusetts Medical Society Prize and the Boylston Medical Society Prize. In 1958 he won the Eli Lilly Award of the American Diabetes Association. Doctor Field's investigations have resulted in more than thirty-five published papers.

DISTINGUISHED SERVICE AWARD

From time to time the American Goiter Association honors one of its members with the Distinguished Service Award. This certificate of merit is given to those who have made outstanding contributions to the organization of the Association and who have assisted in promoting the welfare and influence of the Association. The last award was given in 1959. This year the award was made to *Elmer C. Bartels, M.D.* of Boston, Massachusetts.

Doctor Bartels was born in Ohio. He received his undergraduate and medical training in the University of Illinois. Postgraduate studies were carried out in the Western Pennsylvania Hospital, Pittsburgh and at the

Mayo Clinic, Rochester, Minnesota. He received the M.S. degree in Medicine from the University of Minnesota Medical School in 1932. From 1935 to the present Doctor Bartels has been a member of the staff of the Lahey Clinic.

Doctor Bartels has contributed hundreds of papers to the medical literature, most of which have dealt with various aspects of thyroid gland disease and gout. He has won awards for exhibits on both of these subjects from the American Medical Association.

A past-president of the American Goiter Association, Doctor Bartels has also served as a committee member or committee chairman, where he has wisely used his influence to improve the organization. His contributions to strengthening the Association and making its influence world-wide have been almost as numerous as his scientific additions to medical literature.

Doctor Bartels is married and their family consists of four children and grandchildren.

Inter-Society Cytology Council, Inc.

The Annual Scientific Meeting of the Inter-Society Cytology Council will be held in the Hotel Peabody, Memphis, Tennessee, *November 2, 3, and 4, 1961*. Dr. Paul A. Younge, Secretary-Treasurer, 1101 Beacon Street, Brookline 46, Massachusetts. Everyone interested in cytology is welcome to attend.

International Symposium on Radioactive Isotopes in Clinical Medicine and Research

The Fifth International Symposium on Radioactive Isotopes in Clinical Medicine and Research, arranged by the Second Medical University Clinic, Vienna (Director Prof. Dr. K. Fellinger), will take place in Bad Gastein, Austria, *January 9–12, 1962*. The papers to be discussed will be restricted to the following topics:

1. Computer and Analogue Techniques for the Evaluation of Tracer Data.

2. Recording of Rapidly Changing Counting Rates.
3. Use of Radioactive Gases in Clinical Studies.
4. Clinical and Laboratory in Vitro Techniques.
5. Studies of Absorption of Labeled Compounds in Gastroenterology.
6. Tracer Techniques in Renal and Liver Function Studies.
7. Studies of Cell Population Kinetics, Including Quantitative Autoradiographic Studies.

Applications to present papers should be submitted *before July 30, 1961*, and should be accompanied by a short abstract.

Inquiries and applications should be sent to: Dr. Rudolf Höfer, Radioisotopes Laboratory, Second Medical University Clinic, Garnisongasse 13, Vienna 9, Austria.

Neuroendocrinology Symposium

The Endocrinology Study Section of the National Institutes of Health is sponsoring a Symposium on Neuroendocrinology to be held on *December 6 7 and 8, 1961*, at Miami, Florida. Registration: December 5 (noon to 6:00 p.m.). Opening meeting: December 6, 9:00 a.m. Those interested in attending should address requests for room reservations to:

Neuroendocrinology Symposium,
The New Everglades Hotel,
Miami, Florida.

By special arrangement single rooms will cost $8.00, rooms with twin beds and double occupancy, $12.00. These rates will also prevail for a period before and after the meeting, and the hotel management should be contacted for details. For information on the symposium and for a program, write to A. V. Nalbandov, 102 Animal Genetics, University of Illinois, Urbana, Illinois. Total attendance at the meeting will be limited to about 250 persons and reservations for rooms will be accepted by the hotel on a first-come, first-served basis after May 1.

PROGRAM

1. Prologue
   Speaker: R. T. Hill

2. Hypothalamic afferents (limbic system, reticular formation, afferents from midbrain, cortical afferents)
   Speaker: W. J. H. Nauta
   Discussant: P. D. MacLean
3. Vascular connections between hypothalamus, posterior and anterior pituitary gland
   Speaker: J. Landsmeer
   Discussant: H. Duvernoy
4. Neurohypophyseal secretions and their origin
   Speaker: W. H. Sayer
   Discussants: S. W. Smith
   T. F. Leveque
5. Central nervous system and the secretion and release of ACTH
   Speaker: W. F. Ganong
   Discussants: R. H. Egdahl
   E. M. Anderson
6. CNS and the secretion and release of TSH
   Speaker: S. A. D'Angelo
   Discussant: G. W. Harris
7. CNS and the secretion and release of LH and FSH
   Speaker: B. Flerkó
   Discussants: C. A. Barraclough
   G. W. Harris
8. CNS and the secretion and release of prolactin
   Speaker: J. Meites
   Discussants: S. J. Folley
   A. S. Parkes
   H. N. Bruce
9. Physiology of the pituitary gland as affected by transplantation or stalk transection
   Speaker: J. W. Everett
   Discussants: R. K. Meyer
   R. Courrier
   V. Critchlow
   A. V. Nalbandov
10. Recent advances in the chemistry of neuroendocrine chemical mediators originating in the CNS
    Speaker: R. Guillemin
    Discussants: J. C. Porter
    S. D. McCann
11. Role of peripheral nerves in the function of gonads, accessories and duct systems
    Speaker: R. M. Melampy
    Discussants: R. T. Hill
    A. V. Nalbandov
12. Role of light on the neuroendocrine system
    Speaker: V. Critchlow
    Discussant: A. Wolfson
13. Pharmacology of neuroendocrine blocking agents
    Speaker: P. L. Munson
    Discussant: C. H. Sawyer

14. Human neuroendocrinology
    Speaker: C. Lloyd
    Discussants: A. E. Rakoff
    I. A. Mirsky
15. Concluding Session: Synthesis and summary of symposium
    Speaker: R. O. Greep

Book Register

Biosynthese und Stoffwechselwirkungen der Schilddrüsenhormons. By Doz. Dr. Ing. Günther Hillmann, Dozent für physiologische Chemie, und Leiter des Laboratoriums der medizinischen Universitätsklinik, Tübingen. Monographien No. 8, Biochemie und Klinik by G. Weitzel and N. Zöllner (in German). Part I deals with the biosynthesis and metabolism of iodinated amino acids and the relationship between thyroid diseases and changes in iodine metabolism. Part II covers the metabolic action of thyroxine *in vivo*—its effect on metamorphosis, water and mineral metabolism, protein, carbohydrate and fat metabolism, oxygen consumption, thermoregulation, lactation and the cardiovascular and central nervous systems; special stress is laid on the relation between thyroid function and enzyme activity, and on the relation between chemical constitution of the hormone and biologic action. Part III is devoted to the metabolic actions of the thyroid hormone *in vitro*. Part IV covers the modern chemical, isotopic and biologic procedures for the measurement of thyroidal substances; paper; 133 pages; 3 figures; 7 tables; 562 references; 1961. Georg Thieme Verlag, Stuttgart. In the U.S.A. and Canada: Intercontinental Medical Book Corporation, New York 16, N.Y. Price DM 26.50; $6.30.

Developmental Genetics and Lethal Factors. By Ernst Hadorn, Professor of Zoology and Comparative Anatomy, University of Zurich. Translated by Ursula Mittwoch, University College, London. This text presents basic information on the origin of lethal factors by mutation and upon their role in the production of developmental abnormalities. After a consideration of concepts and terms, the subjects covered are: methods of demonstrating lethal factors; permanent culture of lethal factors; balanced systems; the origin of lethal factors by mutation; the chromosomal basis of lethal mutants; dominant lethal factors; polygenic lethality; maternal influence and cytoplasmic effects; degrees of penetrance and expressivity; dominance and recessivity of action; phase specificity of action; cell and organ specific action; pleiotropic pattern of action and damage; experiments on cellular autonomy (transplantation and tissue culture); phenocopies; biochemical abnormalities in lethal mutants; physiologic aspects of abnormal development; possibilities of therapy; economic losses; and general remarks; 355 pages; 129 figures; 27 tables; extensive bibliography; 1961. Methuen and Company, Ltd., London, England; John Wiley and Sons, Inc., New York. Price $8.50.

Die Intersexualität. Edited by PROF. DR. CLAUS OVERZIER, Chief Physician, The Medical Clinic, University of Mainz and with contributions by the following collaborators: M. L. BARR, London, Canada; J. R. BIERICH, Hamburg; W. M. DAVISON, London, England; C. E. FORD, Harwell, England; H. GELBKE, Göttingen; R. HASCHE-KLÜNDER, Hanover; G. A. HAUSER, Basel; H. D. HENNING, Cologne; K. HOFFMAN, Mainz; W. KOCH, Munich; B. LENNOX, Glasgow; J. M. OPITZ, Iowa City; J. H. SCHULTZ, Berlin; D. R. SMITH, Reading, England; M. WATZKA, Mainz; E. WITSCHI, Iowa City; and J. ZANDER, Cologne. (In German.) The 20 chapters of this well documented book, representing the work of 18 leading investigators in the field, cover the following subjects: Normal development of the gonads and genital ducts; the basis of intersexuality (genetic and post-genetic); intersexuality in mammals; sex chromatin in various animals, in embryos, *in vitro*, and post mortem; origin of sex chromatin, and morphology, with applications to clinical diagnosis; sex chromatin in the nuclei of leukocytes; cytogenesis of intersexuality in man, (including Klinefelter syndrome, Turner syndrome, Triplo-X women, XXXY forms, true X-defect, XO/XXX mosaic); hormones and intersexuality; (including schema and methods for estimation of steroids and gonadotropins, with application to clinical syndromes); research aspects (clinical, laboratory, psychologic); true hermaphrodism; pseudohermaphrodism; testicular geminization; the so-called "true" Klinefelter syndrome; gonadal dysgenesis; the adrenogenital syndrome; induced pseudohermaphrodism; tumors with heterosexual activity (detailed tables); nuclear anomalies in tumor cells; plastic operations; genital malformations without intersexuality; intersexuality and transvestism (biology and psychology); 560 pages; 193 figures in 447 parts; 38 tables; bibliography with each chapter; 1961. Price DM 119; $28.35.

Enzyme Regulations in Clinical Medicine. Proceedings of the Sixth International Congress of Internal Medicine, edited by A. GIGON and H. LUDWIG, Basel, Switzerland. The papers are in English, German or French. The Congress was held in Basel in August 1960, under the auspices of the International Society of Internal Medicine. The importance of enzymes in biochemistry, physiology and clinical medicine is emphasized in 17 papers and a panel discussion. Topics of the various papers include enzyme regulation mechanisms; biochemical report on enzyme regulations in the clinic; gene and enzyme; biochemical activity and the mechanical function of the heart; cardiac metabolism and enzymes; enzyme regulations in the kidneys; blood enzyme activities in liver disease; bilirubin and liver enzymes; enzymatic regulation of the liver; enzymes in hematology; metabolic error and thyroid disease; congenital hypothyroidism through defective dehalogenation of iodotyrosines; muscle enzymes and pathologic conditions of the muscles; enzymic regulations in the bones; 3 papers on the diagnostic value of enzymes; therapeutic indications for enzymes; and 3 papers on mucoviscidosis (cystic fibrosis); paper; 261 pages; 77 figures; 18 tables; 1961. Benno Schwabe and Company, Publishers, Basel, Switzerland. In the U.S.A. and Canada: Intercontinental Medical Book Corporation, New York 16, N.Y. Price $7.00.

Nicht-glykolytische Stoffwechselwege der Glucose. By PROF. DR. S. HOLLMANN, Physiologische-chemisches Institut, University of Göttingen. Monographien No. 10, Biochemie und Klinik, by G. WEITZEL and N. ZÖLLNER (in German). The subjects covered are: the primary reactions of glucose in metabolism; the

products of these reactions (D-gluconic acid, sorbitol, and glucose-6-phosphate) and their fate; the pentose phosphate cycle with its regulation, defects and functions; the degradation of glucose; the metabolism of glucuronic acid and ascorbic acid; the metabolism of pentoses; the relation between galactose metabolism and glucose metabolism; the synthesis of amino sugars and their derivatives; the metabolism and function of nucleosiddiphosphoglycosyl compounds; the metabolism of L-fucose and L-rhamnose; clinical aspects; and methods for isolation, identification and estimation of sugars and their derivatives; paper; 220 pages; 51 figures; 29 tables; 1138 references; 1961. Georg Thieme Verlag, Stuttgart. In the U.S.A. and Canada: Intercontinental Medical Book Corporation, New York 16, N.Y. Price DM 39; $9.30.

Oxosteroids: The Use of Phenolic Hydrazides for Detection, Characterisation and Estimation. By BERNARD CAMBER, M.D., Research Fellow, Neuro-Psychiatric Research Institute of the West of Scotland, Fellow of the Medical Research Council, Advanced Medical Fellow of the Commonwealth Fund, Visiting Investigator and Fellow of the Rockefeller Institute, Research Fellow of the College of Physicians and Surgeons of Columbia University and Research Associate, Charing Cross Hospital. This monograph is based upon the thesis presented for the degree of Doctor of Medicine, University of Glasgow. Part I deals with the advantages and limitations of chemical methods currently in use for the detection, identification and qualitative and quantitative measurement of oxosteroids in body fluids and tissues; the need for improved reagents is stressed. Part II covers preliminary investigations with various hydrazides. Part III is devoted to 2-hydroxy-3-naphthoic acid hydrazide, Part IV to β-resorcylic acid hydrazide, and Part V to salicylic acid hydrazide. The salicylol hydrazide and the indoaniline reactions constitute the most important discoveries in this study; 79 pages; 8 figures; 12 tables; 1960. H. K. Lewis and Company, Ltd., London, England. Price 12 s. 6 d. net.

Pathogenesis and Therapy in Edema. Proceedings of the Sixth International Congress of Internal Medicine, edited by A. GIGON and H. LUDWIG, Basel, Switzerland. The papers are German, English or French. The Congress was held in Basel in August 1960, under the auspices of the International Society of Internal Medicine. The clinic on edema covers the mechanism of edema formation in congestive heart failure, various aspects of aldosterone metabolism (5 papers), the role of the kidney, regulatory factors in edema, "fluid lung" in anuria-oliguria, and analbuminemia. A panel discussion on nephrosis covers electron microscopic studies of renal vascular disease in childhood, experimental nephrosis, nephrotic edema, blood volume and glomerular permeability to proteins, differential diagnosis of the nephrotic syndromes in adults, immune globulins, and the management of the child with nephrosis. A panel discussion on interstitial nephritis covers the definition and frequency of nephritis, chronic pyelophritis, acute toxic interstitial nephritis, the toxicology of phenacetin, abuse of phenacetin and erythrocyte survival, and chronic unilateral ascending nephropathy in the rabbit. A panel discussion on diuretics covers the mechanism of action of the new diuretic compounds, electrolyte disturbances in cirrhosis and heart failure, diabetes insipidus, renal debt and diuresis, the influence of chlorothiazide and hygroton on the peripheral circulation, and dosage schedules for the new diuretics; paper; 206 pages; 88 figures; 17 tables, 1961. Benno Schwabe and Company, Publishers, Basel, Switzerland. In the U.S.A. and

Canada: Intercontinental Medical Book Corporation, New York 16, N.Y. Price $5.00.

Radioisotope Techniques in Clinical Research and Diagnosis. By N. VEALL, B.Sc., Guy's Hospital Research Fellow; Honorary Research Physicist, Institute of Obstetrics and Gynecology, University of London, and N. VETTER, M.D., Head, Radioisotopes Laboratory, Second Medical University Clinic, Allgemeines Krankenhaus, Vienna, Austria. Fundamental principles of isotope methods and instrumentation in research are covered in detail, and applications of radioactive isotopes to clinical diagnosis are outlined. Subjects discussed in separate chapters are: radioactive isotopes; statistical factors affecting measurements; radiation detectors; electronic equipment; radioactive measuring techniques; multiple tracer techniques; radiation hazards; dosimetry; radiochemical procedures; simple dynamic systems; body composition and electrolyte studies; blood volume; survival of transfused red cells; iron metabolism; vitamin B_{12}; thyroid function tests and localization studies; plasma protein turnover studies; intestinal absorption studies with I^{131}-labeled fat and protein; circulation studies, localization and differential diagnosis of malignant tumors, and applications and limitations of isotope therapy; 417 pages; 74 figures; tables; 1958. Butterworth and Company (Publishers), Ltd., London, England, and Washington D. C. Price $10.00.

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ANNUAL MEETING • NEW YORK, N.Y. • June 22, 23 & 24, 1961

Prof. Dr. H. U. ZOLLINGER, Zürich

Die interstitielle Nephritis

Mit einem Vorwort von Prof. Dr. H. v. Meyenburg

VIII + 264 p., 87 fig., 3 tab., 1945. US $7.—

Inhalt:

Einleitung — Interstitielle Nephritis bei bakteriellen Erkrankungen (a. Infektionskrankheiten, b. Infektiöse Prozesse) — Interstitielle Nephritis bei Eiweiß-Stoffwechselstörungen (a. Akut, b. Chronisch) — Interstitielle Nierenveränderungen bei Harnstauung; ascendierender, nicht-eitriger Nephritis — Tumorartige Infiltrationsprozesse des Niereninterstitiums — Interstitielle Begleitnephritis — Interstitielle Nephritis bei Tier (a. Spontannephritis, b. Experimentelle Untersuchungen) — Nomenklatur und Einteilung der interstitiellen Nierenprozesse — Normale und Patho-Histologie des Niereninterstitiums — Pathogenese der interstitiellen Nephritis — Zusammenhänge zwischen klinischen und anatomischen Befunden — Beitrag zur Therapie der Anurie bei interstitieller Nephritis — Zusammenfassung — Literatur.

Die primär nur für den Pathologen interessante interstitielle Nephritis ist in den letzten Jahren vor allem in ihrer chronischen Form zu einem in zahlreichen Ländern beobachteten Problem geworden. Insbesondere wird ihr Zusammenhang mit einem Phenazetin-Abusus heute von zahlreichen Seiten bestätigt. Die vorliegende Monographie gibt vor allem Auskunft über die Entwicklung der akuten Formen, ihre Pathogenese, Abgrenzung und ihre pathophysiologischen Folgen.

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THE MECHANISM OF ACTION OF INSULIN *edited by* **W. A. Broom,** *Boots Pure Drug Co., Ltd., Nottingham, and* **F. W. Wolff,** *Post Graduate Medical School, London.* Seventy-seven chemists and clinicians met in London for a symposium on insulin. The resulting vigorous discussion covered: Chemical Nature of Insulin; Insulin and Transport Systems; Insulin and Intermediary Metabolism; Insulin and the Liver; Insulin and Protein Synthesis; Bio-Assay of Insulin; Insulin Antagonists; Mechanism of Action of Insulin and Other Hypoglycaemid Substances; Clinical Aspects of Diabetes and Pre-Diabetes. **Pub. date Oct. '60, 336 pp., 128 il., 60 tables, $7.50**

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DIABETES MELLITUS: A Clinical and Pathological Study of 2529 Cases *(A Carl V. Weller Lecture). By* **E. T. Bell,** *Univ. of Minnesota.* The striking beneficial influence of insulin in prolonging the life of young diabetics is emphasized. Covers also: Development of atherosclerosis in diabetics, hyalinization of the islets of Langerhans, hyalinization of the juxtaglomerular segment of the afferent renal arterioles, diagnosis by examination of the kidneys, and histological determination of the insulin content of the pancreas. **Pub. date Oct. '60, 80 pp., 8 il. (1 in full color), (Amer. Lec. Medicine), $3.75**

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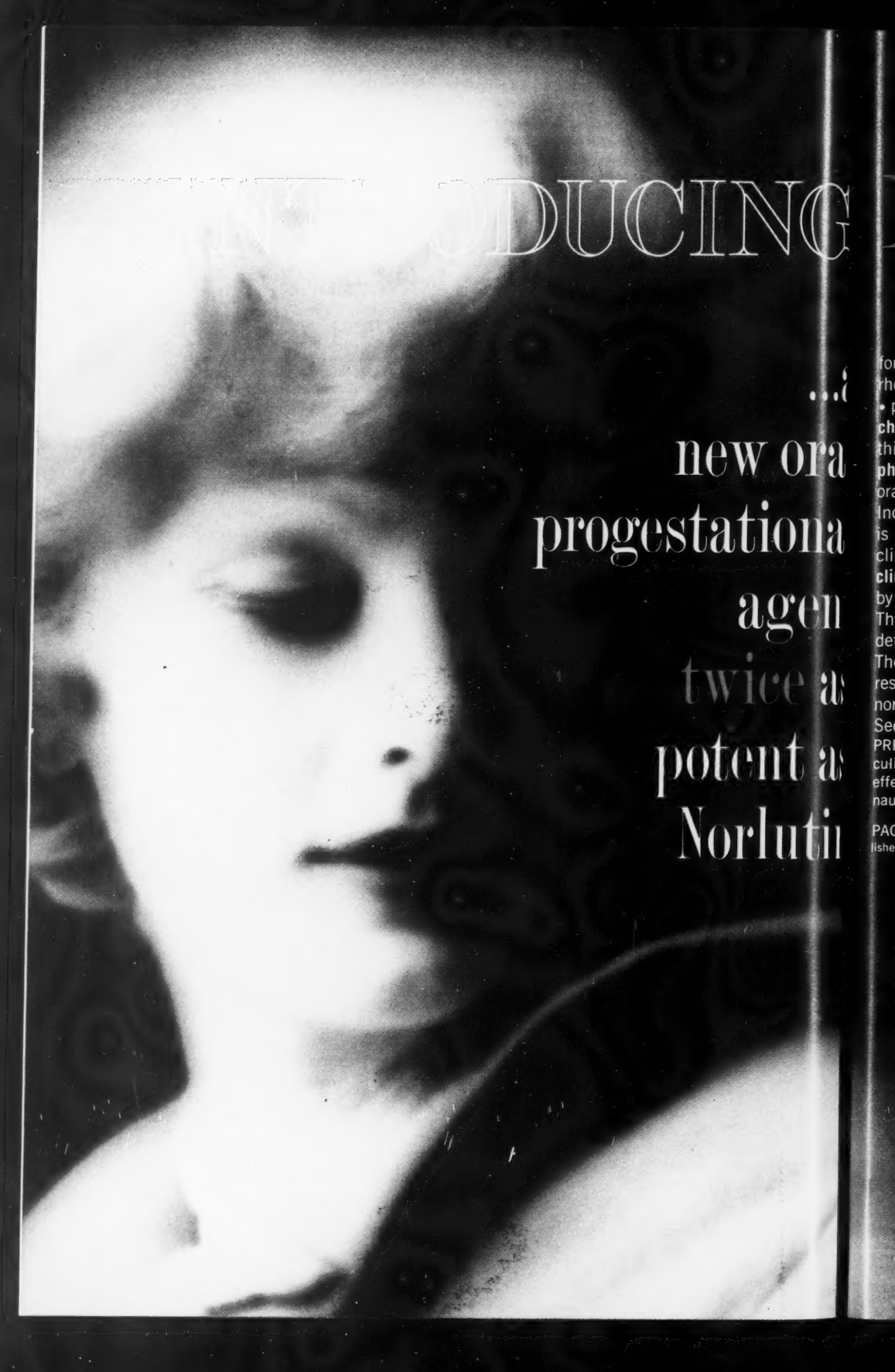
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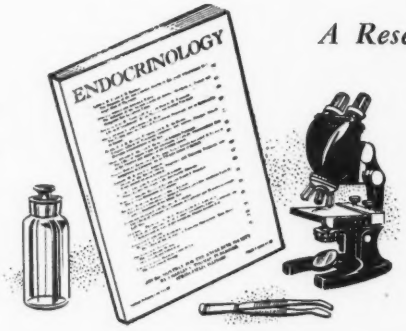

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